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# DISSECTING THE ROLE OF AMYLOID FIBRIL DEPOSITION IN THE KIDNEY IN FAMILIAL AMYLOIDOTIC POLYNEUROPATHY

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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No cumprimento do Decreto-Lei supra mencionado, a autora desta tese declara que interveio na concepção e execução do trabalho experimental, assim como na interpretação e discussão dos resultados e na sua redação.

Outros artigos publicados pela autora durante o seu doutoramento que, não tendo sido usados nos resultados desta tese, estão no âmbito do tema aqui desenvolvido:

Lacerda PC*, **Moreira L***, Vitorino R, Costa PP. Use of MALDI-TOF Mass Spectrometry to Assay the Transthyretin V30M Mutation in Serum From a Liver Transplant Donor: A Case Report. Transplantation (in press).

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## Abbreviations most frequently used in the text

AGEs – advanced glycation end products
AL – immunoglobulin amyloidosis
ATF3 – activating transcription factor 3
ATTR amyloidosis – amyloidosis caused by transthyretin amyloid deposition
ATTRV30M – transthyretin with a methionine-for-valine substitution at position 30
ATTRV30M amyloidosis - amyloidosis caused by the mutant transthyretin with a methionine-for-valine substitution at position 30
CNS – central nervous system
CSF – cerebrospinal fluid
Ct – threshold cycle
DN – Diabetic nephropathy
DMOG – dimethyloxalylglycine
DLS – Dynamic Light Scattering
EPO – erythropoietin
EPOR – EPO receptor
ER – endoplasmic reticulum
ERK – extracellular signal-regulated kinase
FACS – fluorescence-activated cell sorting
FAP – Familial Amyloidotic Polyneuropathy
FBS – fetal bovine serum
FIH1 – HIF inhibition factor
GAG – sulfated glycosaminoglycans
GATA-2 – GATA binding protein 2
GATA-4 – GATA binding protein 4
HEK293T – human embryonic kidney 293 cell line
Hep3B – human hepatocellular carcinoma
HIF-1 – hypoxia inducible factor 1
HIF-2 – hypoxia inducible factor 1
HNF-4 – hepatocyte nuclear factor 4
HRE – hypoxia response element
HUVECs – primary human umbilical vein endothelial cells
IHC – Immunohistochemistry
IL-1β – interleukin-1β
IMAC – immobilized metal ion affinity chromatography

iNOS – inducible nitric oxide synthase
IPTG – Isopropyl β-D-1-thiogalactopyranoside
JAK2 – Janus kinase 2
LT – liver transplantation
MAPK – mitogen-activated protein kinase
MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB – nuclear factor-κB
NO – nitric oxide
PDGF – platelet-derived growth factor
PHDs – prolyl-hydroxylases
qPCR – quantitative PCR
RAGE – receptor of advanged glycation end products
RBP – retinol binding protein
RFLP – restriction fragment length polymorphism
ROS – reactive oxygen species
RPC – renal progenitor cells CD133+CD24+
RPE – human retinal pigment epithelial cells
RT-PCR – real time PCR
SAA – senile systemic amyloidosis
SAP – Serum Amyloid P component
SD – standard deviation
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH-SY5Y – human neuroblastoma cell line
siRNAs – small interfering RNAs
T4 – thyroxine hormone
TBP – TATA-binding protein
TFA – trifluoroacetic acid
ThT – thioflavin T
TNF-α – tumour necrosis factor-α
TTR – transthyretin
TTRV30M – transthyretin with a methionine-for-valine substitution at position 30
wt-TTR – wild-type TTR

# Resumo

A Polineuropatia Amiloidótica Familiar (PAF) ou amiloidose ATTRV30M é uma doença neurodegenerativa, autossómica dominante, causada pela deposição de amilóide extracelular de transtirretina mutante (TTRV30M), afectando principalmente o sistema nervoso periférico. É caracterizada por uma polineuropatia periférica sensitivo-motora progressiva e disfunção autonômica, com manifestações renais, cardíacas e oculares.
A anemia afecta cerca de 25% dos doentes PAF sintomáticos e é caracterizada por uma produção ineficiente de eritropoietina (EPO), independentemente da presença de insuficiência renal. Precede por vezes a doença clínica, sugerindo um bloqueio das células renais produtoras de EPO. Excluímos anteriormente um efeito inibitório dos depósitos de amiloide fibrilar e da TTRV30M em circulação, mas o papel dos agregados de TTR não-fibrilares na produção de EPO renal ainda precisava ser explorado. Os agregados não-fibrilares ou oligómeros de TTR são citotóxicos, induzindo stress oxidativo e a expressão de moléculas relacionadas com a apoptose, e a secreção de citoquinas pró-inflamatórias. Alguns destes marcadores também são capazes de inibir a produção de EPO. A expressão do gene EPO é regulada ao nível da transcrição pelos factores de transcrição HIF, NF-kB e GATA-2. O HIF induz a expressão de EPO em condições de hipóxia, por ligação ao enhancer na região 3', enquanto o GATA-2 e NF-kB inibem a sua expressão por ligação ao promotor na região 5' do gene.

Neste trabalho propusémo-nos explorar os mecanismos moleculares envolvidos no bloqueio da produção de EPO na PAF. Utilisando diferentes modelos de cultura de células, foi avaliada a influência dos agregados oligoméricos de TTR na viabilidade celular, capacidade de diferenciação, expressão do gene da EPO e atividade do seu promotor.
Oligómeros de TTR foram preparados envelhecendo a proteína a pH fisiológico, seguido de 5 minutos de agitação. As preparações que continham predominantemente espécies de 300 nm foram usadas para os ensaios celulares.
Uma redução modesta mas estatisticamente significativa da viabilidade celular foi induzida pela TTRV30M oligomérica após 24 horas de incubação, independentemente do tipo de célula. Concomitantemente com a redução da viabilidade, foi observado um aumento da actividade das caspases 3/7 em células Hep3B, SH-SY5Y e RPE expostas aos agregados oligoméricos de TTRV30M. Estes resultados estão de acordo com estudos anteriores realizados noutros outros tipos de células, e mostram que a apoptose está implicada na perda de viabilidade celular.

A influência dos agregados oligoméricos sobre a diferenciação celular foi avaliada em células progenitoras renais (RPC). Estas têm potencial de diferenciação em podócitos e células tubulares renais. Embora os oligómeros de TTRV30M tenham inibido a proliferação das RPC, eles não influenciaram a sua capacidade de se diferenciar em podócitos funcionalmente maduros, e, portanto, não devem comprometer a regeneração dos tecidos.

A expressão do gene da EPO foi avaliada por PCR em tempo real. Células Hep3B e RPE tratadas com TTRV30M oligomérica mostraram uma redução significativa (aproximadamente 50%) da expressão de EPO após 24 horas em condições de normóxia quando comparado com o controlo e com a exposição à forma tetramérica. Os oligómeros de TTR normal também reduziram a expressão de EPO em 22% em células Hep3B em normóxia, quando comparada com a com a exposição à forma tetramérica. Estes resultados apoiam a nossa hipótese de que espécies oligoméricas citotóxicas estarão envolvidas na génese da anemia em doentes com PAF. Além disso, mostrámos recentemente que as concentrações de EPO no humor aquoso de olhos com glaucoma de doentes não-PAF estão significativamente aumentadas relativamente a olhos sem glaucoma. No entanto, em olhos com glaucoma de doentes PAF os níveis de EPO não aumentam e mantêm níveis semelhantes aos dos olhos normais, o que mostra a incapacidade destes doentes regularem positivamente a produção ocular de EPO.

Um ensaio repórter foi construído com um fragmento de PCR do promotor da EPO, que contém os locais de reconhecimento para GATA-2 e NF-kB, ligados ao gene da luciferase para avaliar o papel dos factores de transcrição do promotor. Células Hep3B transfectadas e expostas durante 24 horas a TTRV30M oligomérica mostraram uma redução significativa da actividade do promotor de EPO (53%) quando comparado com o controlo e exposição à forma tetramérica. Estes resultados sugerem que a expressão de EPO é inibida pelos agregados oligoméricos de TTRV30M, pelo menos em parte através da inibição da actividade do promotor.

Imunofluorescência e imunohistoquímica foram realizadas para explorar o envolvimento do NF-kB e GATA-2 na redução da atividade do promotor da EPO, mas não foram observadas diferenças significativas entre as células tratadas com oligómeros ou com a forma tetramérica de TTR.

Em conclusão, os agregados de TTR não-fibrilares podem inibir a produção de EPO e contribuir para o aparecimento precoce da anemia nos doentes PAF. Estudos adicionais são necessários para elucidar os mecanismos que levam à inibição da EPO, a fim de proporcionar marcadores úteis para a avaliação do doente e, eventualmente, novos alvos terapêuticos. As células RPE, sendo produtoras quer de TTR como de EPO, poderão constituir um bom modelo para estes estudos futuros.

# Abstract

ATTRV30M amyloidosis or Familial Amyloidotic Polyneuropathy (FAP) is a neurodegenerative, autosomal dominant disease, caused by the extracelular amyloid deposition of a mutant transthyretin (TTRV30M), affecting particularly the peripheral nervous system. It is characterized by progressive sensorimotor peripheral polyneuropathy and autonomic dysfunction, and renal, cardiac, and ocular manifestations. Anemia affects about 25% of symptomatic FAP patients and courses with low erythropoietin (EPO) levels, independently of the presence of renal failure. It sometimes precedes clinical disease, suggesting a blockage of kidney's EPO-producing cells. We had previously excluded an inhibitory effect of the amyloid fibril deposits and of circulating TTRV30M, but the role of early non-fibrillar TTR aggregates on renal EPO production still needed to be explored. Early non-fibrillar TTR aggregates are highly cytotoxic, inducing oxidative stress and the expression of apoptosis-related molecules, and secretion of pro-inflammatory cytokines. Some of these markers are also capable of inhibiting EPO production. EPO gene expression is regulated at the transcriptional level by HIF, NF-kB and GATA-2 transcription factors. HIF induces EPO expression under hypoxic conditions, by binding to the enhancer in the 3' region, whereas GATA-2 and NF-kB inhibit its expression by binding to the promoter in the 5' region of the gene.

In this work, our aim was to explore the molecular mechanisms involved in the blockage of EPO production in FAP. Using different cell culture models, we assessed the influence of oligomeric TTR aggregates on cell viability, differentiation capacity, EPO gene expression and promoter activity.

TTR oligomers were prepared by aging the protein at physiological pH, followed by 5 minutes of stirring. Preparations containing species mainly of 300 nm were used for the cell culture-based assays.

A modest but statistically significant reduction in cell viability was induced by oligomeric TTRV30M after 24 hours of incubation, independently of the cell type. Concomitantantly with the reduction of cell viability, an increase in caspase 3/7 activity was seen in Hep3B, SH-SY5Y and RPE cells exposed to the oligomeric TTRV30M aggregates. These results agree with those of previous studies performed with other cell types, and implicate apoptosis in the loss of viability.

The influence of oligomeric aggregates on cell differentiation was evaluated on renal progenitor cells (RPC). RPC have self-renewal and multidifferentiation potential into podocytes and renal tubular cells. Although TTRV30M oligomers inhibited RPC

proliferation, they did not influence their capacity to differentiate into functionally mature podocytes, and thus should not compromise tissue regeneration.

EPO mRNA expression was evaluated by real-time PCR. Hep3B and RPE cells treated with oligomeric TTRV30M showed a significant reduction (about 50%) of *EPO* mRNA expression after 24 hours in normoxia, when compared to the control and cells exposed to the tetrameric form. Oligomers from normal (wild type) TTR also reduced *EPO* expression by 22% in normoxic Hep3B cells when compared to exposure to the tetrameric form. These results support our hypothesis that cytotoxic oligomeric species are involved in the genesis of anemia in FAP patients. Besides this evidence, we recently showed that EPO concentrations in the aqueous humor of glaucomatous eyes of non-amyloidotic patients are significantly increased relatively to normal non-glaucomatous eyes. However, in glaucomatous eyes of FAP patients the EPO levels did not increase and maintained similar levels to those of control eyes, showing an inability of these patients to upregulate ocular EPO production.

A reporter assay was constructed with a PCR fragment of the EPO promoter, containing the recognition sites for GATA-2 and NF-kB linked to the luciferase gene, to evaluate the role of transcription factors targeting the promoter. Transfected Hep3B cells exposed for 24 hours to oligomeric TTRV30M showed a significant reduction of the erythropoietin promoter activity (53%) when compared to the control and exposure to the tetrameric form. These results suggest that EPO expression is inhibited by oligomeric TTRV30M aggregates, at least in part through inhibition of promoter activity.

Immunofluorescence and immunohistochemistry were performed to explore the involvement of NF-kB and GATA-2 in the reduction of EPO promoter activity, but no significant differences were observed between the oligomeric TTR-treated and tetrameric TTR-treated cells.

In conclusion, early non-fibrillar TTR aggregates can inhibit EPO production and may contribute to the early onset of anemia in these patients. Further studies are needed to elucidate the mechanisms that lead to EPO inhibition, in order to provide useful markers for patient evaluation, and possibly new targets for therapeutic development. RPE cells, as producers of both TTR and EPO, could be a good model for these future studies.

# INTRODUCTION

# 1. Amyloid and amyloidosis: short story of its discovery

Amyloid is an insoluble substance that deposits in tissues and organs, mainly in the extracellular spaces, leading to progressive organ dysfunction and disease [1-3]. Amyloidosis is the group of diseases associated with amyloid deposition [4]. Different types of amyloidosis exist, depending on the protein that originates the fibrillar deposits.
Some proteins undergo conformational changes of their structure due to abnormal polymeric assemblies of its subunits, and form amyloid fibrils. Amyloid deposits are composed by non-branching fibrils with a β-sheet structure and approximately 10 nm in diameter [4]. The fibrils bind the dye Congo red and exhibit green birefringence when the Congo red-stained deposits are viewed with polarized light.

## 1.1. Historical review

The historical review of the discovery of amyloid presented here had as major bibliographic sources the detailed accounts of Sipe JD *et al*. [5] and Kyle RA *et al.* [6].

The first description of what we now call amyloidosis may have occured in 1639, when Nicolaus Fontanus reported the autopsy of a young man who had an abscess in the liver and a large spleen filled with white stones, probably a “sago spleen” amyloidosis.
In 1838, Matthias Schleiden, a German botanist, used the term amyloid to describe a normal amylaceous constituent of plants. Later, in 1854, Rudolph Virchow used the same term to describe the corpora amylacea of the nervous system and the substance implicated in lardaceous degeneration. He found that, using iodine, these stained blue, turning violet upon the subsequent addition of sulfuric acid. This peculiar reaction made Virchow consider that these lardaceous deposits were identical to starch.
In 1859, Carl Friedreich and August Kekule saw that this amyloid “mass” had a proteic nature instead of carbohydrate, unlike amylon or cellulose. From this point, amyloid has been considered a protein material and, later, as a group of proteins with a propensity to undergo conformational changes that result in the formation of fibrils. The name amyloid prevailed nonetheless.

Nowadays, it is known that amyloid deposits in tissues have also other non-fibrillar components besides the protein, such as proteoglycans (heparan sulfate or chondroitin sulfate type), basement membrane constituents (laminin, fibronectin and collagen IV),

serum amyloid P component, sulfated glycosaminoglycans (GAG) and apolipoprotein E [7-9]. Although the mechanisms by which these components interact with the amyloid fibrils are not fully understood, evidence suggests that they may influence the amyloid structure to assume a beta pleated sheet rather than an alpha helical conformation [10-11]. Also, they may contribute to amyloidogenesis by increasing fibril stability, delaying their clearance and protecting the amyloid peptide from proteolytic breakdown [12].

In 1922, a new method to detect the presence of amyloid deposits was introduced and is still used today. Bennhold injected Congo red, a metachromatic cotton wool dye, in patients with amyloid. He noted the disappearance of the dye from the plasma and its accumulation in amyloid tissue. Later, in 1927, Divry and Florkin described independently a particular property of amyloid stained with Congo red: the apple-green birefringence. Amyloid plaques, when stained with Congo red and visualized under polarized light, exhibited positive birefringence with respect to the long axis of the deposits, as a result of Congo red intercalating into the fibrils. This property of congophilia with apple green birefringence was adopted as the main criterion to define the amyloid substance. The typical fibrillar morphology is the second criterion.

In 1959, Cohen and Calkins characterized the structure of amyloid [1]. Using electron microscopy, they recognized that all types of amyloid shared a similar non-branching fibrillar ultrastructure in fixed tissue sections. Later, X-ray diffraction analyses showed that amyloid fibrils were ordered with the polypeptide backbone configured as a beta pleated sheet and oriented perpendicular to the fibril axis [1-3, 13].
In 1967, Shirahama and Cohen saw that the diameter of the isolated amyloid fibril was approximately 75-80 Å. A pair of amyloid protofibril about 25-35 Å wide were arranged along the long axis of the fibril in a slow twist [14], with an outer band of 4-75 Å and a inner band of 8-9 Å [13].

In 1971, Benditt and Glenner characterized the biochemical heterogeneity of amyloid. Using amino acid sequence determination, they saw that each unique protein was associated with particular clinical syndromes. They first reported that primary amyloidosis was the result of the deposition of fragments of immunoglobulin light chains (AL) either of λ- or κ-type [15-16], and that secondary amyloidosis in patients with chronic and recurrent acute inflammatory diseases was the result of the deposition of an unknown protein, named AA [17-18].

Research on amyloidosis has evolved and nowadays several proteins are known to undergo conformational changes causing different amyloid diseases, each with its unique clinical features [19].

### 1.2. Classification of the amyloidosis

A large number of unrelated proteins are known to form amyloid *in vivo*. A nomenclature has been developed to classify the amyloidosis according to the chemical identity of the amyloid fibril forming protein.

In 1975, Thomas *et al.* presented a broad classification of the amyloidosis according to the anatomical system that is predominantly affected [20]. This classification has been continuously updated and nowadays is managed by the Nomenclature Committee of the International Society of Amyloidosis (ISA). According to the last data, there are 31 known extracellular fibril proteins in humans, two of which are iatrogenic in nature (table 1) [4].

The nomenclature is based on the amyloid fibril protein, that is designated with a preffix A, followed by a suffix that is an abbreviated form of the precursor protein name [4]. The amyloidosis syndromes are named after the amyloid fibril protein, e.g. AL amyloidosis, wild-type ATTR amyloidosis or hereditary ATTRV30M (p. TTRV50M) amyloidosis.
Besides this precise molecular classification, amyloidosis are divided into systemic or localized and in primary, secondary and inherited amyloidosis. This classification is important for clinical practice as the patient's therapies and prognoses are different [21].

A list of known amyloid fibril proteins, and their precursors, which form extracellular deposits in humans is given on table 1. Some proteins, instead, form intracellular amyloid inclusions (table 2).

Table 1 - Amyloid fibril proteins and their precursors in human. From Sipe JD *et al.* [4].

| Fibril protein | Precursor protein | Systemic and/or localized | Acquired or hereditary | Target organs |
|---|---|---|---|---|
| **AL** | Immunoglobulin Light Chain | S, L | A, H | All organs except CNS |
| **AH** | Immunoglobulin Heavy Chain | S, L | A | All organs except CNS |
| **AA** | (Apo) Serum Amyloid A | S | A | All organs except CNS |
| **ATTR** | Transthyretin, wild type | S | A | Heart mainly in males, ligaments, tenosynovium |
| | Transthyretin, variants | S | H | PNS, ANS, heart, eye, leptomen |
| **Aβ2M** | B2-Microglobulin, wild-type | L | A | Musculoskeletal system |
| | B2-Microglobulin, variant | S | H | ANS |
| **AApoAI** | Apolipoprotein A I, variants | S | H | Heart, liver, kidney, PNS, testis, larynx (C terminal variants), skin (C terminal variants) |
| **AApoAII** | Apolipoprotein A II, variants | S | H | Kidney |
| **AApoAIV** | Apolipoprotein A IV, wild type | S | A | Kidney medulla and systemic |
| **AGel** | Gelsolin, variants | S | H | PNS, cornea |
| **ALys** | Lysozyme, variants | S | H | Kidney |
| **ALECT2** | Leukocyte Chemotactic Factor-2 | S | A | Kidney, primarily |
| **AFib** | Fibrinogen α, variants | S | H | Kidney, primarily |
| **ACys** | Cystatin C, variants | S | H | PNS, skin |
| **ABri** | ABriPP, variants | S | H | CNS |
| **ADan*** | ADanPP, variants | L | H | CNS |
| **Aβ** | Aβ precursor, wild-type | L | A | CNS |
| | Aβ precursor, variant | L | H | CNS |
| **APrP** | Prion protein, wild type | L | A | CJD, fatal insomnia |
| | Prion protein, variants | L | H | CJD, GSS syndrome, fatal insomnia |
| **ACal** | (Pro)calcitonin | L | A | C-cell thyroid tumors |
| **AIAPP** | Islet Amyloid Peptide† | L | A | Islets of Langerhans, Insulinomas |
| **AANF** | Atrial Natriuretic Factor | L | A | Cardiac atria |
| **APro** | Prolactin | L | A | Pituitary prolactinomas, aging pituitary |
| **AIns** | Insulin | L | A | Iatrogenic, local injection |
| **ASPC‡** | Lung Surfactant Protein | L | A | Lung |
| **AGal7** | Galectin 7 | L | A | Skin |
| **ACor** | Corneodesmosin | L | A | Cornified epithelia, hair follicle |
| **AMed** | Lactadherin | L | A | Senile aortic, media |
| **AKer** | Kerato-epithelin | L | A | Cornea, hereditary |
| **ALac** | Lactoferrin | L | A | Cornea |
| **AOAAP** | Odontogenic Ameloblast-Associated Protein | L | A | Odontogenic tumors |
| **ASem1** | Semenogelin 1 | L | A | Vesicula seminalis |
| **AEnf** | Enfurvitide | L | A | Iatrogenic |

*ADan is the product of the same gene as ABri.; †Also called amylin.; ‡Not proven by amino acid sequence analysis.

Table 2 - Intracellular inclusions with known biochemical composition, with or without amyloid properties. From Sype JD *et al.* [4].

| Inclusion name | Site | Protein nature | Examples of associated disease |
|---|---|---|---|
| **Lewy bodies** | Neurons intracytoplasmic | α-synuclein* | Parkinson's disease |
| **Huntington bodies** | Neurons intranuclear | Polyq expanded Huntingtin | Huntington's disease |
| **Hirano bodies** | Neurons | Actin | Neurodegenerative disorders |
| **Collins bodies** | Neurons | Neuroserpin | Forms of familial presenile dementia |
| **Not specified** | Neurons, many different cells | Ferritin | Form of familial neurodegenerative disorder |
| **Neurofibrillary tangles** | Neurons intracytoplasmic | Tau | Alzheimer disease, fronto-temporal dementia, aging, other cerebral conditions |

### 1.3. Primary *vs* secondary and localized *vs* systemic amyloidosis

Amyloidoses may be either idiopathic (primary form) or associated with certain inflammatory disorders, immunodeficiency states, endocrinopathies or cancer (secondary forms) [22].

In terms of the location of the amyloid deposits, amyloidosis may be localized or systemic. While in localized forms the synthesis of the amyloid precursor and deposition of the fibrils occur within the same organ, in systemic forms of amyloidosis, the fibril precursor protein is synthesized at a particular site, secreted into circulation and transported to different sites [23].

### 1.4. Diagnosis of amyloidosis

Amyloid typing is very important to define the treatment, prognosis and course of the disease.

In cases of hereditary amyloidosis, in which a family history exists and a specific mutation is considered, genetic testing may be performed. Techniques such as DNA sequencing, PCR followed by restriction fragment length polymorphism (RFLP) or high resolution melting are commonly used to confirm the diagnosis.

In cases of acquired amyloidosis or in the absence of a clear family history, the diagnosis is usually based on the detection of amyloid deposits in tissue biopsies. Congo red staining is performed to detect amyloid deposition, revealed by the presence of the characteristic apple-green birefringence (figure 1). When amyloidosis is confirmed, it is necessary to type it. Immunohistochemistry on paraffin sections or immunofluorescence of frozen sections are techniques routinely performed using a panel of antibodies recognizing different amyloid proteins [21, 24]. Immunoelectron microscopy sometimes is also used. These antibody based techniques are relatively simple and routinely used in pathology laboratories. The results are generally reliable but these techniques also have some drawbacks. Some antibodies have low affinity for the misfolded amyloid proteins. Also, some mutations may cause loss of epitopes and consequently absence of antibody binding [25-26].

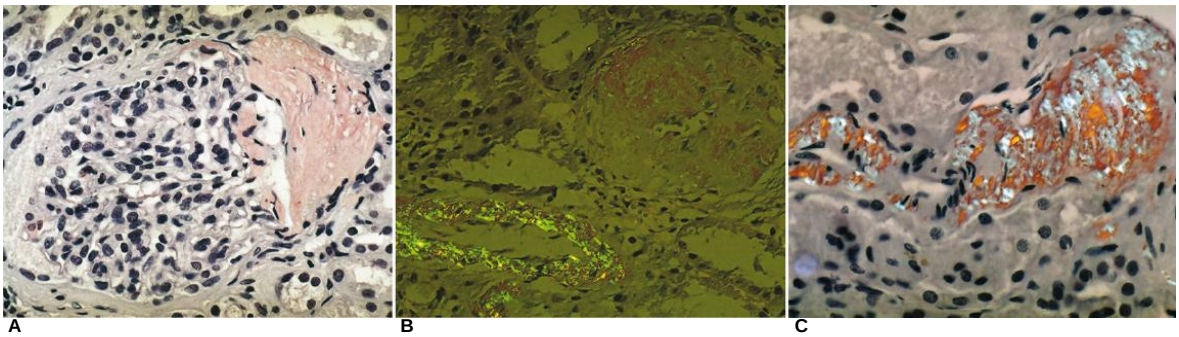


Figure 1 – Congo red staining of renal biopsies of ATTR amyloidosis, showing amyloid deposition in glomeruli, vasculature, and tubulointerstitium. (A) Congo red staining visualized under light microscope; (B) Congo red staining visualized under polarized light, showing the apple-green birefringence; (C) Higher magnification of Congo red staining visualized under polarized light. From Lobato *et al.* [27].

When doubts persist, amyloid fibril proteins can be extracted and amino acid sequence analysis performed [28-29]. Proteomic analysis, particularly mass spectrometry, has become a useful tool for amyloid typing. Fibrils may be characterized by two-dimensional gel electrophoresis (2D-PAGE). Laser microdissection of Congo red positive areas from formaline fixed paraffin tissues can also be carried out. Samples are then subjected to tandem mass spectrometric analysis to identify the amyloid protein [30]. The recent multidimensional protein identification technology (MudPIT) enables a faster identification from a peptide mixture of a protein sample. It is a chromatography-based technique that couples a triphasic microcapillary column (reversed phase, strong cation exchange and reversed phase high-performance liquid chromatography grade materials) with a tandem mass spectrometer [31-33]. The peptide mixture is loaded directly onto the triphasic microcapillary column which is placed directly in-line with the tandem mass spectrometer. Ideally, these methods should complement each other to allow for an unequivocal result.

Among all the amyloidogenic proteins, in the following sections attention will be given to a systemic and hereditary form of amyloidosis, the ATTR amyloidosis or Familial Amyloidotic Polyneuropathy (FAP).

# 2. Familial Amyloidotic Polineuropathy or ATTR amyloidosis

Transthyretin (TTR) is one of the precursor proteins associated with systemic amyloidosis. More than 100 mutations have been described for TTR [34-35] and most of them, as well as wild type TTR itself, are amyloidogenic. Only about 10 known TTR mutations show no propensity to cause amyloidosis.

Deposition of wild-type TTR is associated with an acquired form of amyloidosis, senile systemic amyloidosis (SAA). It affects particularly the heart, leading to cardiac complications, and is found mainly in older individuals (>60 years old) and predominantly in men. SSA affects approximatly 25% of the population aged more than 80 years [36-37].

Amyloidogenic mutations are responsible for hereditary diseases that share some clinical manifestations, mainly peripheral and autonomic neuropathy, in familial amyloid polyneuropathy (FAP), and less commonly cardiopathy, in familial amyloid cardiopathy.

## 2.1. ATTRV30M amyloidosis

Historically named Familial Amyloidotic Polyneuropathy type I (FAP-I) or Portuguese type, ATTRV30M amyloidosis is a neurodegenerative, autosomal dominant disease, characterized by extracelular deposition of mutated TTR (V30M) derived amyloid fibrils [38].

As a matter of simplicity, throughout this document the historical term FAP will be used to refer to ATTRV30M amyloidosis.

FAP was first described in 1952 by Corino de Andrade. He reported several patients, the first one observed in 1939 in the Santo Antonio Hospital in Oporto, who had a peculiar form of peripheral neuropathy with atypical generalized amyloidosis, or paramyloidosis, with special involvement of the peripheral nerves [39].

In 1978, Costa *et al.* [40] identified TTR, then known as thyroxin-binding prealbumin, as the major constituent of the amyloid deposits in these patients. In 1984, Saraiva *et al.* identified a substitution of methionine for valine at position 30 of the TTR protein as the biochemical cause of Portuguese type FAP [38]. Today it is known that Val30Met is the most frequent disease associated TTR mutation in Portugal, Sweden, Japan and Italy. Although being considered a rare disease (it affects approximately 1:100.000 persons worldwide), ATTRV30M amyloidosis is an endemic disease particularly prevalent in the

northern regions of Portugal, mainly Póvoa do Varzim, where allele frequency is approximately 1:550 [36, 41].

### 2.2. Clinical features of ATTRV30M amyloidosis

ATTRV30M amyloidosis is a systemic disease characterized by progressive sensorimotor peripheral polyneuropathy and autonomic dysfunction, with renal, cardiac, and ocular manifestations, among others [42]. Usually symptoms begin in the third to fourth decade of life, and develop gradually for 10-20 years, leading to death. Progressive impairment of thermal sensitivity and pain are common initial symptoms. The disease progresses with lowering of the general state of health, alimentary and sexual dysfunction, malabsorption, urinary bladder dysfunction, abnormal glomerular function, cardiac insufficiency and vitreous opacities [43-46]. Besides the peripheral nerves, where the amyloid is preferentially deposited causing myelin sheet destruction, other organs, such as kidney, pancreas, heart, stomach, aorta, skin and eye are affected [47]. Although TTR is produced mainly by the liver, this organ is not significantly affected.

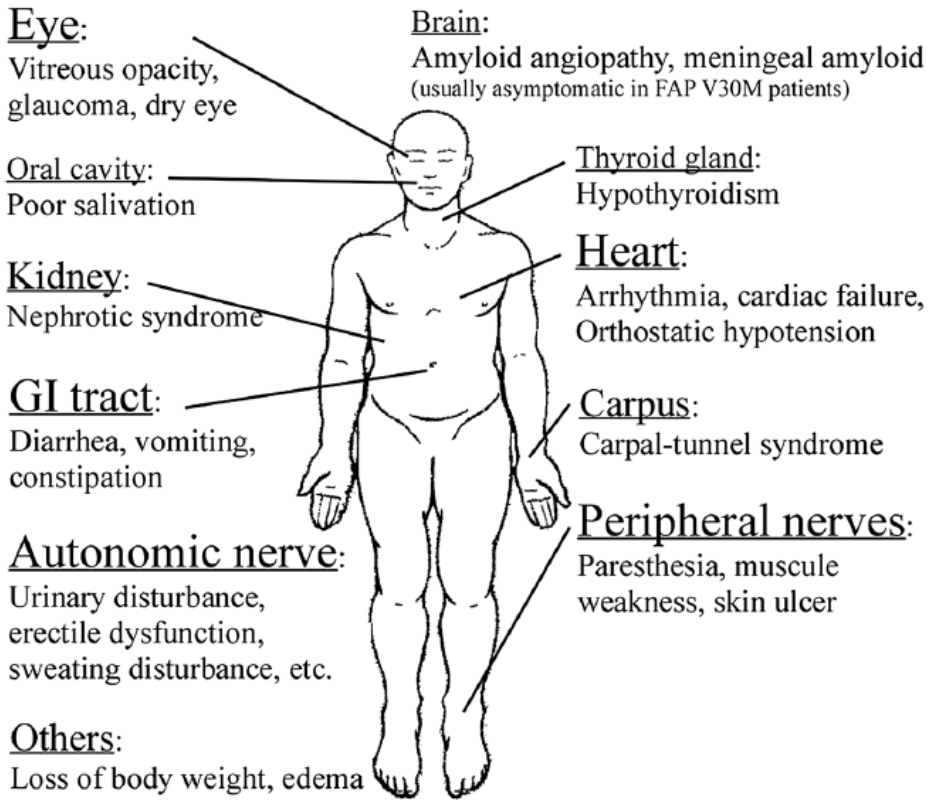


Figure 2 – Schematic representation of clinical manifestations in FAP patients. From Ueda *et al.* [47].

In this work, more attention will be given to renal and ocular complications, and in particular, to the expression of erythropoietin by these two organs in FAP patients. This subject will be discussed later in further detail.

### 2.3. Therapeutic strategies for ATTR amyloidosis

Advances are being made in the treatment of ATTR amyloidosis and many therapeutic strategies have been proposed, which act on 3 important checkpoints:

A) Blocking the synthesis of TTR protein
B) Stabilization of the TTR tetramer to inhibit TTR disaggregation
C) Disruption and promotion of TTR amyloid fibrils clearance

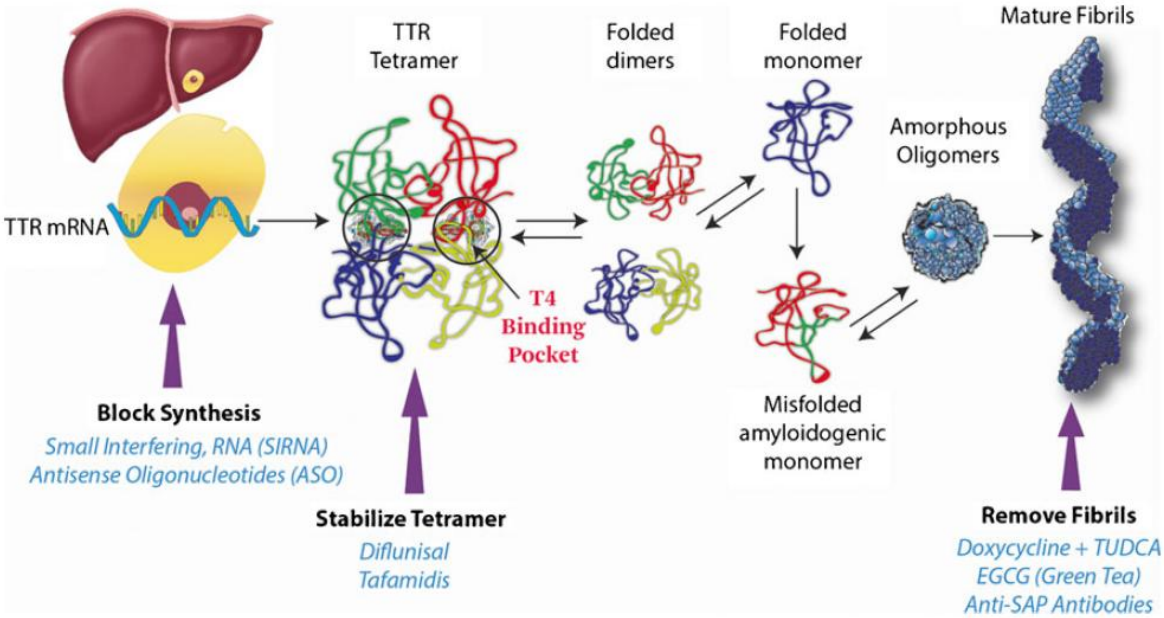


Figure 3 – Schematic representation of the mechanism proposed for TTR amyloidogenesis and some therapeutic strategies for FAP. From Hanna 2014 [48].

- **<u>Block TTR synthesis</u>**

**Liver transplantation**

Since 1990, liver transplantation (LT) has become a therapeutic option for FAP patients, as it eliminates more than 95% of the abnormal protein from circulation [49-50]. Approximately 120 LTs are performed worldwide each year [http://www.fapwtr.org]. When LT is performed in the early stage of the disease, it can prolong survival, halt the

progression of clinical manifestations and even improve autonomic and, to a lesser extent, peripheral nerve function [51-53].

A strategy to manage the scarcity of healthy livers for transplantation was adopted with sequential or domino LT, in which FAP patients receive a healthy liver from a deceased donor and their liver is transplanted into patients with malignant or end-stage liver diseases. As FAP only manifests after the 2$^{nd}$ or 3$^{rd}$ decade of life, it would be expected that a FAP liver recipient would not manifest the disease. However, some recipients of FAP livers started developing TTR amyloid deposits and disease symptoms less than 10 years after the surgery [54-56].

LT has important drawbacks: it is extremely expensive; transplanted patients must be treated with immunosuppressants lifelong; non-symptomatic carriers of TTR mutations as well as FAP patients in advanced stage do not undergo liver transplantation. Although circulating mutant TTR is virtually eliminated, there are reports of continuing formation and deposition of fibrils from wild-type TTR, causing cardiomyopathy and/or neuropathy after liver transplantation, findings that seem similar to those observed in senile systemic amyloidosis (SSA) [57-60]. Also, LT does not prevent the production of mutant TTR in the cerebrospinal fluid and in the eyes, where the mutant protein is still secreted by choroid plexus and retinal pigmented epithelium, respectively. Ocular and central nervous system (CNS) problems may occur, highlighting the importance of non-neural dysfunction in post-transplant patient management [61-62].

**Gene therapy**

Antisense oligonucleotides (ASO) and small interfering RNAs (siRNAs) are effective gene-silencing tools that could be promising for FAP gene therapy [48]. Blocking hepatocyte synthesis of TTR would prevent the production of both mutant and wild type TTR, which could be a potential treatment for both hereditary and acquired ATTR amyloidosis. Phase 3 studies are currently ongoing for some ASOs and siRNAs [48, 63].

**Antisense oligonucleotides (ASOs)** are synthetic single stranded oligomers designed to be complementary to a specific region in a target mRNA, promoting its degradation, thus preventing production of the associated protein [64]. The drug ISIS-TTR Rx from Isis Pharmaceuticals (Carlsbad, CA) targets the TTR mRNA preventing the production of both mutant and wild-type TTR protein. This ASO suppresses TTR mRNA levels in the liver and in the choroid plexus of the brain [65-67].

**siRNAs** are sequence-specific post-transcriptional gene silencing molecules. Patisiran, also known as ALN-TTR02, from Alnylam Pharmaceuticals (Cambridge, MA) is a lipid nanoparticle formulation of a synthetic siRNA that selectively silences both mutant and wild-type TTR gene expression, both *in vitro* and *in vivo* [65, 68-69].

- **Stabilize the TTR tetramer**

TTR binding of its natural ligand thyroxine (T4) stabilizes the tetramer against dissociation. The same strategy has been tested using small molecules that could bind to the TTR T4 pocket to kinetically stabilize the native tetramer and avoid the conformational changes that lead to amyloid fibril formation [70].

**Diflunisal,** a non-steroidal anti-inflammatory drug (NSAID) with a molecular structure similar to T4, binds to the T4 binding sites on the TTR tetramer, stabilizing it and preventing acid mediated fibril formation *in vitro* [71-74], as well as in serum of amyloidosis patients, without adverse effects [73, 75]. A 2 years long clinical trial showed that Diflunisal treated patients had substantially less polyneuropathy progression compared to placebo [76].

**Tafamidis** (Vyndaqel) or Fx-1006A, like Diflunisal, is a small molecule that selectively binds to the T4 binding site and stabilizes the TTR tetramer [77], but without NSAID activity. FAP patients treated with Tafamadis have shown a significant benefit relatively to an untreated control group. Tafamidis stabilizes plasmatic TTRV30M protein, slowing disease progression [77-78]. It became the first pharmacological treatment for FAP patients to be approved in Europe [79].

- **Clearance of TTR amyloid fibrils**

The presence of other compounds in the amyloid fibrils besides the core protein led several groups to investigate the possibility to target them as a strategy to promote clearance of amyloid fibrils, regardless of the amyloid type.

**Doxycycline**, an antibiotic, was shown to be an effective fibril disrupter, disaggregating TTR amyloid fibris in mice transgenic for human TTRV30M, promoting amyloid deposit reabsorption [80-81].

**TUDCA** (Tauroursodeoxycholic acid), a biliary acid with antioxidant and antiapoptotic activities, significantly decreased the amount of TTR aggregates, as well as oxidative and apoptotic biomarkers associated with disease, also in a transgenic mouse model [82-83].

A trial of a combination of TUDCA with Doxycyline was conducted in aged FAP mice [82] and was more effective than either doxydoxycycline or TUDCA alone. It significantly lowered TTR deposition and associated tissue markers and also disaggregated mature amyloid deposits in the gastrointestinal tract.

**EGCG** (Epigallocatechin-3-gallate), the predominant polyphenol in green tea, has shown *in vitro* the capacity to both inhibit fibril formation and also disrupt amyloid fibrils by converting existing fibrils into non-fibril conformers [84-86].

**Anti-SAP antibodies** could contribute to clear amyloid fibrils. Serum Amyloid P component (SAP) is a plasma glycoprotein universally associated with amyloid fibrils. Encouraging data was seen in animal models as well as in a heterogeneous group of amyloid patients treated with anti-SAP antibodies [87-88].

### 2.4. Diagnosis

As for other amyloidosis, the diagnosis is based on histological examination, genetic testing or mass spectrometry. ATTRV30M amyloidosis results from a single adenine for guanine nucleotide change in exon 2 [89], which creates a restriction site for the enzyme *NsiI*. This facilitates the molecular diagnosis, which can be performed by PCR followed by RFLP, or by real-time PCR (rtPCR) genotyping based on melting curve analysis. Also, amino acid substitution results in a known mass shift in the protein molecular weight [90], making it possible to use mass spectrometry analysis [91-93]. This is particularly useful in the setting of liver transplantation.

Recently, our group described a case report of a FAP patient who underwent orthotopic liver transplantation from a cadaveric donor [94]. Continuing deterioration in this patient raised the suspicion, confirmed in 2012, that the liver donor was also a TTRV30M carrier, so retransplantation was proposed and carried out. Immunoprecipitation of TTR from the serum of the cadaveric donor, followed by mass spectrometry analysis [95] enabled to confirm the absence of TTRV30M, concluding that this second time the transplanted liver was FAP free.

# 3. Renal and ocular complications in ATTRV30M Amyloidosis: its association with low erythropoietin production

TTRV30M amyloid deposits are commonly found both in the kidney as well as in the eye of FAP patients and give rise, respectively, to renal and ocular problems in these patients.

## 3.1. Renal complications: nephrotic syndrome and anemia

In ATTRV30M amyloidosis, renal amyloid deposition occurs mainly in the glomerular mesangium and medulla, with a typical involvement of the distal convoluted tubule and Henle's loop, which may lead to tubular atrophy and loss of tubular function [27]. Clinical nephropathy manifests initially as a microalbuminuria and then progresses to proteinuria and renal failure. In FAP patients, nephrotic syndrome may be associated with anemia with decreased levels of EPO, sometimes below the lower limit of the normal range, without associated iron deficit [96]. These patients do not respond to iron therapy but treatment with recombinant EPO is effective [97].
According to World Health Organization criteria, anemia is defined as a concentration of hemoglobin (Hb) <13 g / dL in men and <12 g / dL in women [98].

### 3.1.1. Defective EPO production by the kidney in ATTRV30M amyloidosis

Anemia has been described in ATTRV30M amyloidosis patients:

- Moderate normocytic normochromic anemia was observed in 39% of the Swedish FAP patients [99];
- macrocytic and hypochromic anemia was reported in a group of 35 Japanese FAP patients [100].
- normocytic and normochromic anemia was present in 25% from a total of 165 symptomatic FAP patients, even in the presence of normal renal function, and is associated with a defective renal production of EPO, revealed by serum levels lower than expected [96].

A deficit of EPO in Portuguese FAP patients is an early event that was observed independently of the presence of renal failure and sometimes preceding clinical disease

[96]. Serum EPO levels were, on average, 11.2+6.7 mU/mL, lower than the expected levels (35+13.9 mU/ml) for the degree of anemia, while iron stores, B12 vitamin, and serum folate levels were normal in these patients. Normalization of iron status was insufficient for the correction of anemia, but therapy with low doses of recombinant EPO was effective [97], excluding a defective response of the bone marrow as a cause of anemia in these patients.

Circulating EPO is mainly produced by the kidney in the adult. The observed low EPO production suggests a defect of the EPO-producing cells, which could be related to either the presence of amyloid deposits in the renal interstitium or with other factors, such as circulating TTRV30M itself.

The amyloid deposits present in renal biopsies were found to have no correlation with serum EPO levels, independently of the neuropathy score, the amount of amyloid deposition or the renal clinical manifestations [101].

Anemia in liver transplant recipients is usually due to the side-effects of immunosuppressive therapies, iron deficiency, renal failure and post-transplant lymphoproliferative disorders [102]. A study performed in cirrhotic patients showed that anemia affected 85% of these patients before liver transplantation. After liver transplantation, anemia decreased to 18% [103]. In contrast, in FAP patients the prevalence of anemia increased even after liver transplantation, and defective endogenous EPO production persisted [104], excluding an inhibitory effect of the circulating TTRV30M on the EPO-producing cells.

Pro-inflammatory cytokines can inhibit EPO gene expression, contributing to the anemia of chronic disease [105]. In ATTRV30M amyloidosis, inflammation is observed, particularly with up-regulation of TNF-α, macrophage colony-stimulating factor and IL-1β [106-107], which could explain the low EPO levels in these patients. However, Beirão *et al.* analyzed 24 FAP patients and found no evidence of systemic inflammation, as no significant differences were found on interleukin-6, transferrin saturation, ferritin and hepcidin-25 [108].

### 3.2. Ocular complications: vitreous opacities and glaucoma

ATTRV30M amyloidosis is associated with several ocular manifestations such as lacrimal dysfunction, pupillary disturbances, changes in the conjunctiva, presbyopia [109], dry eye [110], vitreous opacities, which may occur before any other systemic manifestation of the disease [111], and, most seriously, severe glaucoma. Liver transplantation in FAP patients

proved unable to halt the progression of these ocular manifestations, probably due to the continued production of the mutated TTR by the retinal pigment epithelial cells [62].
A retrospective study of 477 symptomatic FAP patients was performed by Beirão et al. which showed that these patients have amyloid deposits in the iris, in the anterior lens capsule and in the vitreous. Vitrectomy with complete removal of the vitreous is usually carried out, but when the vitrectomy is incomplete new amyloid deposits are formed due to continuing deposition in the remaining vitreous [112].
Glaucoma can develop rapidly in FAP patients and, if not treated, may lead to blindness. A correlation was found between vitrectomy and glaucoma, with vitrectomy favoring the onset or worsening of glaucoma [113].

### 3.2.1. Defective EPO production by the eye in ATTRV30M amyloidosis

Glaucoma causes an increase in intraocular pressure, which leads to activation of neuroprotective mechanisms. Studies have reported upregulation of EPO expression and an increased intravitreal EPO concentration in some ocular disorders [114], which may reflect the cytoprotective function of EPO in response to hypoxia, ischemia, and inflammation [115]. Recently, our group found that EPO concentrations in the aqueous humor of glaucomatous eyes of non-FAP patients are significantly increased relatively to normal non-glaucomatous eyes, probably as a protective role. However, in glaucomatous eyes of FAP patients the EPO levels did not increase and maintained similar levels to those of control eyes [116]. These results show an inability of FAP patients to upregulate EPO production both systemically by the kidney and locally by the pigmented epithelium.
Glaucoma is the second leading cause of blindness worldwide [117]. Vascular abnormalities and altered blood flow at the optic nerve head may lead to local hypoxia, accelerating neuronal cell death in patients. The hypoxia inducible factor 1 (HIF-1) is thought to be involved in the pathology of glaucoma, as increased activation of HIF-1 was found in glaucomatous eyes and localization of this protein was correlated with regions of visual field defects [118]. HIF is one of the main regulators of EPO expression, by inducing it in situations of hypoxia. In glaucoma, as a consequence of HIF activation, EPO levels are strongly elevated, probably as a cytoprotective response.

The mechanisms responsible for the low expression of EPO in FAP patients, whether as a response to anemia, or as a response to ocular damage, as in the case of glaucoma, remain unexplained. What is certain is that these patients do not increase EPO levels in response to stimuli to which a non-PAF patient would respond with an increase in expression of this cytokine/hormone.

# 4. Erythropoietin

Erythropoietin (EPO) is a hormone essential for red blood cell production. A moderate reduction in hemoglobin concentration is sufficient to increase EPO mRNA expression, which occurs within minutes of the onset of hypoxia, reaching a maximum after 6 hours [119-121]. Daily, it stimulates proliferation and differentiation of about $2x10^{11}$ erythroid progenitor cells in the bone marrow, contributing to the control of blood oxygen capacity throughout the body [122].

EPO is an endocrine, paracrine and autocrine hormone. Besides its hematopoietic function, EPO has been shown to be a cytoprotective hormone. Among other effects, EPO antagonizes the activity of pro-inflammatory cytokines, has neuroprotective functions and promotes healing through stimulation of angiogenesis and capillary growth [123].

## 4.1. Erythropoietin structure

Human EPO is a glycoprotein of 30.4 kDa encoded by 5 exons located in chromosome 7 as a single copy gene. Translation of the EPO gene results in a polypeptide chain of 193 amino acids that is cleaved posttranslationaly, both at the N- and C-terminal sites. The secreted protein has 165 amino acids [124].

About 40% of the molecular weight of EPO is due to carbohydrate chains. EPO has 4 glycosilated side chains that are important to its biological function by conferring thermal and structural stability, protection against free radicals, increased plasma half-life and selectivity [122, 125-126]. EPO has 4 α-helices and 2 dissulfide bonds with 3 asparagine N-glycosilation and 1 serine O-glycosilation sites. The sialic acid residues attached to the 4 carbohydrate chains are particularly important for the maintenance of *in vivo* half-life and biological activity [126-127].

## 4.2. Sites of erythropoietin production

During fetal life EPO is produced by the liver, whereas in the adult it is mainly produced by the kidney [128]. The molecular mechanisms underlying this switch are poorly understood, but are thought to involve the transcription factor GATA-4 [129], which is highly expressed by hepatocytes only in the fetal liver. Its inhibition leads to a dramatic reduction in Epo gene transcription in Hep3B cells.

Many efforts were done to identify the renal EPO-producing cells. Evidence has been provided for different locales including: renal glomeruli [130], peritubular interstitial or endothelial cells in anemic mouse [131-132], peritubular interstitial cells in hypoxic monkey [133], tubular epithelial cells [134-135] and proximal tubular cells [136-137]. In 2010, our group identified distal tubular cells and cortical collecting tubules as the major site of EPO production in normal adult human kidneys from patients with ATTRV30M amyloidosis with or without anemia [138]. In 2013 Bussolati *et al.* identified a subset of renal CD133(+)/CD73(+) progenitor cells isolated from the human renal inner medulla, with a mesenchymal phenotype, as a possible source of EPO under hypoxic conditions, via the prolyl hydroxylase-HIF-2α axis [139]. CD133+ progenitors have been identified along the renal nephron [140] which overlaps with the described localization of EPO-producing cells in different segments of the human nephron by in situ hybridization studies. In addition, Nagai *et al.* demonstrated recently in mice that *EPO* mRNA expression occurs in proximal convoluted tubules (PCTs), distal convoluted tubules (DCTs) and cortical collecting ducts (CCDs) under normoxic conditions and in peritubular cells in severe hypoxia [141]. These dissimilarities may result from inter-species differences. Additionally, it is likely that EPO production by different populations of renal cells depend on the varying hypoxic conditions used in the different experimental models.

Apart from the kidney, EPO production has been found also in the brain [142], retina, lung, spleen, bone marrow, in the male and female reproductive organs [143-144], placenta [145] and also in numerous cancer cells [124].

Although EPO is mainly produced by the kidney in the adult, an adequate renal EPO-producing cell line is not available. So, most of the present knowledge of the $O_2$ sensing mechanism that controls EPO expression has been based on *in vitro* studies using the human hepatoma cell lines Hep3B and HepG2, described in 1987 by Goldberg *et al.* as a constitutive and inducible EPO producer, in an oxygen-dependent manner [146].

### 4.3. Erythropoietin functions: hematopoiesis and cellular protection

Recognition that the EPO receptor (EPOR) was expressed in several cells other than the erythroid progenitor cells led to the discovery of the extra-hematopoietic functions of EPO. EPO is a member of the cytokine type I superfamily [147], and is both an endocrine, paracrine and autocrine hormone.

### 4.3.1. Hematopoietic function

EPO major function is to promote survival of EPO-dependent colony-forming unit-erythroid (CFU-E) cells and erythroblasts. EPO synthesis is regulated at the mRNA level, through mechanisms sensitive to oxygen concentration. In response to hypoxia, EPO production increases in a few hours [148], and acts on the erythroid progenitor cells in the bone marrow stimulating their proliferation and differentiation.

EPO binds to the erythropoietin receptor (EpoR) homodimer, which is located on the surface of erythroid progenitor cells, and triggers a conformational change that brings its intracellular domains into close proximity, resulting in transphosphorylation and activation of Janus kinase 2 (JAK2) [124]. Downstream cascades are initiated via different signaling pathways including signal transducer and activator of transcription 5 (STAT5), phosphoinositide 3-kinase (PI3K)/AKT, and mitogen-activated protein kinase (MAPK) via adapter proteins like Src homology containing protein (SHC). These transcription factors translocate to the nucleus and drive or inactivate transcription of genes involved mainly in survival and prevention of apoptosis of erythroid progenitors [149].

EpoR is expressed at the highest level on erythroid progenitor cells and plays a critical role in the regulation of red blood cell production by EPO. However, expression of the EpoR beyond hematopoietic cells raised the possibility that EPO activity associated with survival, proliferation and differentiation of erythroid cells might not be restricted to erythropoiesis.

EPO can also signal via a heterodimeric receptor composed of an EpoR monomer chain and CD131, the β common cytokine receptor [150]. This heterodimeric complex is found in nonerythroid cells and is thought to be involved in nonerythroid effects of EPO [124].

### 4.3.2. Extra-hematopoietic functions: cellular protection

Recent findings have demonstrated that EPO has also a cytoprotective function.

EPO has direct effects on immune cells, endothelial cells, bone marrow stromal cells, as well as cells of the heart, brain, reproductive system, gastrointestinal tract, muscle, kidney, pancreas, and nervous system [150-152]. The nonerythropoietic functions of EPO include: promoting cardiac and central nervous system development, blocking cell death in stroke models, improving learning and memory, regulating angiogenesis, confer protection in ischemia/reperfusion injury of the kidney, liver, heart and myocardial infarction and modulate responses to injuries such as cerebral ischemia, cardiac infarction and retina degeneration [124, 150]. *In vitro* recombinant EPO stimulates the proliferation, mobilization, and differentiation of endothelial progenitor and precursor cells and also enhances endothelial cell viability and survival by blocking apoptosis [124]. EPO also

protects against diabetes in mouse models, through direct JAK2 signaling in pancreatic cells, resulting in cell survival and proliferation, reduced inflammation, and increased angiogenesis in the islets [150]. EPO may act on the regulation of metabolism and obesity and have potential benefits in the treatment of neurologic diseases, mood symptoms and depression.

In the immune system, EPO responds to tissue injury caused by pathogens, trauma and hypoxia in order to maintain a balance between inflammation and anti-inflammation [153]. To limit an uncontrolled inflammatory response, inflammation or hypoxia can trigger EPO expression to inhibit proinflammatory cytokine production (TNF-α and interleukin 6), to increase expression of endothelial nitric oxide synthase (eNOS) and nitric oxide (NO), and block inducible nitric oxide synthase (iNOS) expression [149, 154], to inhibit macrophage activity by blocking NF-κB p65 signaling pathway [155], and delimit the volume of injury by counteracting apoptosis [153].

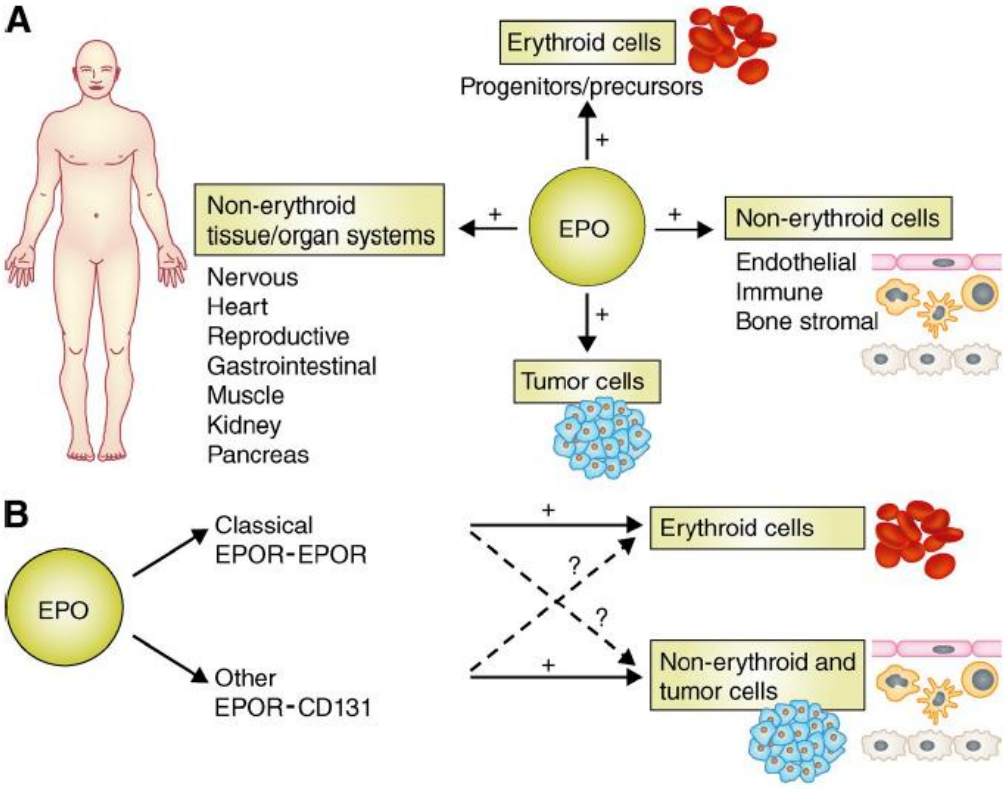


Figure 4 – Multifaceted effects and targets of EPO. (A) EPO targets many cell types and tissues, including erythroid cells and their progenitors, tumor cells, and a variety of other nonerythroid cells and tissues. (B) EPO signals in erythroid cells via EPOR-EPOR homodimers and in nonerythroid cells via EPOR-CD131 heterodimers. From Broxmeyer 2013 [150].

### 4.4. Use of recombinant EPO to treat anemia

A variety of EPO derived recombinant forms of erythroid stimulating agents (ESAs) have been used in research and in clinical practice since 1986 [156]. ESAs are used to treat anemia in several fields such as end-stage renal disease, malignancies associated with chemotherapy, AIDS and surgical interventions [157]. ESAs are able to increase red blood cell count, hemoglobin and hematocrit levels, decreasing the need for red blood cell transfusion and possibly improving quality of life [156].

Several ESAs have been developed, with the rationale of cost saving and prolonged survival in circulation. 'Epoetin' has an amino acid sequence identical to the endogenous human EPO. Darbepoetin alfa has a longer survival in circulation and Mircera (methoxy polyethylene glycol-epoetin beta) has the longest half-life (130-140 h on IV injection) [157].

However, side effects of EPO treatment have emerged: potentially life-threatening cardiac complications such as stroke, increase in arterial blood pressure and possibly hypertension, increase the incidence of thromboembolism [157], especially with higher doses and rapid increase in hemoglobin levels.

### 4.5. Regulation of the erythropoietin gene

EPO gene expression is activated in response to many forms of stress, but hypoxia is the most important stimulus [158]. When $O_2$ concentration drops, EPO gene expression is activated and an exponential increase in EPO protein plasma levels occurs [159].

Regulation of EPO gene expression occurs at different levels: transcription, mRNA stabilization and at the translational level by an upstream open reading frame [160-163].

At the transcriptional level, Hypoxia Inducible Factor (HIF), GATA-2 and NF-kB transcription factors are the main known regulators of EPO gene expression [164-167]. HIF induces EPO expression under hypoxic conditions, while GATA-2 and NF-kB suppress EPO promoter activity when activated by the pro-inflammatory cytokines IL-1 and TNF-α [168].

#### 4.5.1. Hypoxia inducible factor (HIF)

The EPO gene has a cis-acting hypoxia response element (HRE) located in the enhancer at the 3'-flanking region. Under hypoxic conditions, the hypoxia-inducible factor HIF binds to the HRE and induces EPO expression [169, 170].

HIF is a heterodimeric transcription factor composed by an $O_2$-sensitive α-subunit and a constitutively expressed β-subunit, and is expressed in all tissues of many species [171].

Three α chain isoforms of HIF were identified (HIF-1α, HIF-2α and HIF-3α). HIF-1α and HIF-2α have similar regulatory mechanisms and are both involved in $O_2$ delivery and in cellular adaptation to hypoxia. However, several studies point to differences in the expression and cellular distribution pattern, which may be indicative of different functions. Whereas HIF-1α is expressed in all nucleated cells, the expression of HIF-2α is restricted to specific cell types, including vascular endothelial cells, renal interstitial cells, hepatocytes, cardiomyocytes, glial cells and astrocytes [172]. Rosenberger *et al.* found, inducing hypoxia in rats, that regardless of the type of stimulus, the cell populations expressing HIF-1α and HIF-2α in the kidney are different. HIF-1α was predominantly expressed in cells of the proximal and collecting tubules, and HIF-2α was expressed predominantly in the peritubular interstitial cells and some glomerular cells [173]. Because HIF-2α accumulates in peritubular cells and these have been described as the site of EPO production, it was suggested that HIF-2α would be the regulator of EPO expression in the kidney. However, several studies show that both HIF-1α and HIF-2α contribute to the regulation of EPO, although differentially, according to the cell type.

The production and activation of HIF is regulated at the level of the α chain, which increases exponentially as $O_2$ concentration declines. Two oxygen-dependent mechanisms are involved in this regulation. Under normoxia, in the first mechanism, two conserved proline residues located in the oxygen-dependent degradation domain (ODDD) undergo hydroxylation by the action of prolyl-hydroxylases (PHDs) and this allows the ubiquitination of the α chain by the complex von Hippel-Lindau protein (pVHL)/E3-ubiquitin ligase, and subsequent degradation by the proteasome [124]. In the second mechanism, an asparagine residue (Asn803) is hydroxylated by HIF inhibition factor (FIH1) and HIF activity is suppressed by preventing the binding of the transcription coactivator p300/CBP (CREB-binding protein), which acts to increase the expression of their target genes. The PHDs and FIH1 use oxygen as co-substrate. So, under hypoxia, a decrease in the oxygen tension results in inhibition of both PHDs and FIH1. The α-chain dimerizes with the β chain, the HIF complex is stabilized and, after recruitment of co-activator p300/CBP, binds to the HRE of HIF target genes [124]. Additionally, HIF cooperates with hepatocyte nuclear factor 4 (HNF-4), which binds to the 3′ EPO hypoxia enhancer region [124].
Recently, it was shown that HIF-2α may regulate erythropoiesis by a mechanism independent of PHD enzymes. HIF-2α is acetylated during hypoxia and deacetylated by Sirtuin 1, a nicotinamide adenine dinucleotide (NAD)+-dependent protein deacetylase, which increases HIF-2-dependent EPO synthesis, linking cellular redox and energy state to systemic hypoxia responses. Sirtuin 1-deficient mice produced significantly lower

amounts of fetal liver Epo mRNA, and as adults less EPO in response to severe hypoxia [171].

The hydroxylation of HIFα can be carried out by three prolyl-hydroxylases (PHD1, PHD2 and PHD3), among which PHD2 plays the predominant role at most sites. The prolyl hydroxylase inhibitor FG-4497 was used in rats to induce HIF-dependent erythropoietin transcription, and in fact EPO mRNA expression was strongly induced and co-localized with HIF-2α [174]. It is likely that the PHDs are the primary $O_2$ sensors in the control of EPO production. Chemical compounds, such as the α-ketoglutarate, Roxadustat (ASP1517/FG-4592) and dimethyloxalylglycine (DMOG) can inhibit PHDs, providing a potential oral therapy for stimulating erythropoiesis in patients with chronic kidney disease. A phase 3 clinical trial using Roxadustat is currently ongoing to evaluate its safety and efficacy, when compared to epoetin alfa, for the treatment of anemia in patients with chronic kidney disease on dialysis [175-176]. It has the advantage of offering an oral treatment and circunventing the need for multiple injections. DMOG was used in this work to mimick hypoxia conditions and to stimulate *EPO* expression.

### 4.5.2. GATA-2 and NF-kB

Besides HIF, EPO expression is also regulated by GATA-2 and NF-kB at the level of the promoter. There is little information about the mechanisms by which these transcription factors regulate the EPO promoter, but it is known that both act by inhibiting the promoter activity.

The EPO promoter is suppressed by GATA-2 in normoxia and GATA-2 levels decrease in hypoxia [160]. The activation of GATA-2, with consequent inhibition of EPO gene expression, may be related to a pro-inflammatory stimulus, to increased production of $H_2O_2$ and to the presence of L-NG-monomethylarginine (L-NMMA). Exogenous addiction or endogenous production of $H_2O_2$ enhance the expression of GATA-2 and its binding activity, suppressing the activity of the EPO promoter and thus inhibiting EPO gene expression [177-179].

L-NMMA, an endogenous nitric oxide synthase (NOS) inhibitor that is markedly elevated in uremic patients, increases GATA-2 mRNA expression and its binding to the EPO promoter, both in normoxia and hypoxia. EPO promoter activity is inhibited by GATA-2 causing a decrease in EPO expression and protein production [179-180]. L-NMMA did not alter the binding activity of HIF-1, HNF-4 or NF-kB in these experiments.

EPO production may be also suppressed by the pro-inflammatory cytokines IL-1 and TNF-α, which activate GATA-2 and NF-kB and may contribute to the anemia of chronic disease in part by suppressing EPO production [168]. NF-kB binds to the EPO promoter in a

region close to the binding site for GATA-2, and inhibits its activity. Souma T *et al.* showed, with a mouse model of adult-onset anaemia caused by erythropoietin deficiency (ISAM), that inflammatory injury activated NF-kB inducing a pathological phenotypic switch of the renal EPO-producing cells (REP) to myofibroblasts [181].

#### 4.5.3. ATF3

The activating transcription factor ATF3, although not classically described as a regulator of EPO expression, is also involved in the activation of the EPO promoter in response to the platelet-derived growth factor (PDGF) signaling system [182]. ATF3 is rapidly induced upon exposure of cells to stress signals [183]. Elevated expression of PDGF has been observed in RPE cells after retinal detachments or retinal laser treatment in murine model systems [184], in *in vitro* wounded human RPE cell cultures [185] and in epi-retinal membranes isolated from proliferative vitreoretinopathy and proliferative diabetic retinopathy patients [186].

Other transcription factors, such as retinoic X receptor-α (RXR-α), Wilms tumor suppressor (WT1), SMAD3 and Sp1, may contribute to the regulation of EPO expression. RXR-α contributes to EPO activation through interaction with EPO enhancer in the fetal liver during early erythropoiesis [187]. WT1 directly up-regulates EPO expression in hepatocytes, through binding to the EPO promoter [188]. SMAD3 binding to the 3' EPO enhancer, and Sp1 binding to the EPO promoter region also cooperate with HIF, HNF4 and p300 in EPO gene transcription in hypoxia [189]. Together these factors act to stabilize the multifactorial complex interacting with EPO promoter and enhancer to regulate EPO expression.

# 5. The transthyretin protein

Transthyretin (TTR) is a plasma protein involved in the transport of retinol, in a complex with retinol binding protein (RBP), and of the thyroxine (T4) hormone [190-191], hence its name: *trans* (transport) *thy* (thyroxine) and *retin* (RBP). Formerly it was named prealbumin because it migrates just slightly ahead (anodal) of albumin in serum protein electrophoresis.

Liver and choroid plexus are the most abundant sites of TTR synthesis in humans. Liver produces circulating TTR that is secreted to the plasma where it reaches a concentration of approximately 200-250 mg/L (ranging from 3-8 µM) [192-194]. Choroid plexus, in turn, has the highest concentration of TTR mRNA in the body as TTR accounts for 12% of all proteins synthesized [195]. The protein produced by the choroid plexus is secreted into the cerebrospinal fluid (CSF), where it reaches a concentration ranging from 5 to 20 mg/L (0,09-0,4 µM) [193]. In CSF, 80% of T4 is bound to TTR whereas only 15% of T4 is bound to TTR in the serum [196-197].

TTR is also found in the eye, as it is produced by the pigment epithelium (ciliar and retinal) [198], and in less extent in alpha-cells of pancreatic islets [199], yolk sac [200], placenta [201], and intestine [202].

## 5.1. Transthyretin structure

TTR is encoded by a single copy gene with 4 exons located at chromosome 18. Exon 1 codes mainly for a signal peptide of 20 aminoacids that is cleaved before secretion of mature TTR [203-204].

Structurally, TTR is a tetrameric protein with 4 identical subunits, each with 127 aminoacids and a molecular mass of approximately 14 kDa [205]. Each monomer contains 8 β-sheet strands (A-H) and a short helix between strands E and F [206]. The 4 monomers associate non-covalently to form the tetrameric protein, which has a molecular mass of approximately 55 kDa.

Association of two dimers is subjacent to the tetrameric structure. The strength of the interactions between monomers (resulting in a dimer) and between dimers (forming a tetramer) suggests that the dimer rather than the monomer or tetramer is the most stable unit of the TTR structure [207].

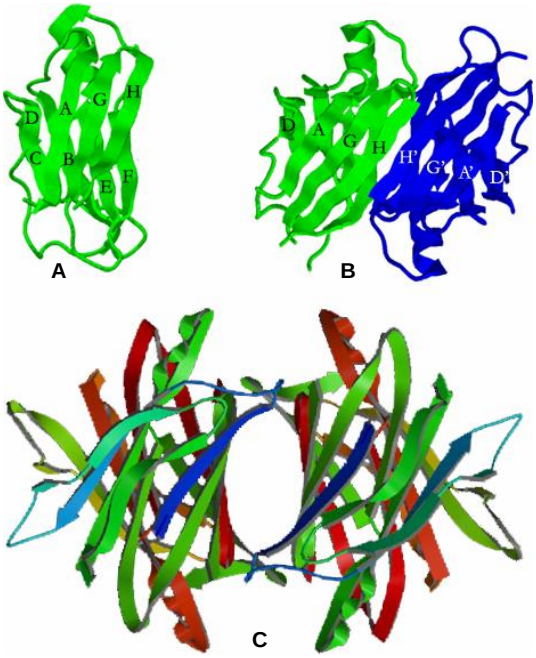


Figure 5 – Structure of human transthyretin monomer (A), dimer (B) and tetramer (C). From Protein Data Bank, PDB ID:s 1F41, 2PAB

The tetramer has 2 identical binding sites for T4 in a central channel and 4 surface binding sites for the complex RBP/vitamin A. However, only one binding site is occupied by T4 under physiological conditions due to negative co-operativity [208]. Also, only one RBP molecule can bind to TTR due to steric hindrance [209].

### 5.2. TTR function

TTR main function is the transport of T4 and of retinol-binding protein (RBP), which in turn transports vitamin A, both in the plasma and in the CSF. However, TTR is not likely to be essential for life or developmentbecause no abnormalities are found in mice that have had the transthyretin gene inactivated [210].

There are studies that suggest that the complex TTR-T4 is endocytosed and internalised. This phenomenon was observed in hepatomas, primary hepatocytes and renal cells and involves an endocytic multi-ligand receptor of the LDL receptor family, megalin (LRP2) [211-213].

TTR also has a protease activity [214]. A fraction of plasma TTR circulates bound to Apolipoprotein A-1 (ApoA1) and acts as a protease, as it is able to cleave the carboxyl-terminal domain, after a phenylalanine residue, of ApoA1 [214]. TTR can also cleave full length β-amyloid to a smaller fragment, and play a protective role in AD [215].

### 5.3. Models of amyloidogenesis

Various mechanisms have been proposed to explain amyloidogenesis.

Mutations in several proteins lead to structural changes that promote unfolding and predispose for the formation of fibers, resulting in hereditary amyloidosis syndromes. In the case of TTR, several mutations alter the thermodynamics and kinetics of dissociation of the tetramer and favor the formation of intermediates that self assemble into amyloid fibers [216].
For example, the V30M mutation confers a moderate instability to the tetramer, while L55P is the most unstable and pathogenic variant. However, the presence of mutations is not essential for amyloidogenesis to be triggered. Examples are some localized amyloidoses, and again using the example of TTR, senile systemic amyloidosis is caused by deposition of amyloid fibers from wild-type TTR. Many factors can trigger destabilization of the protein structure: heat shock, oxidative stress or chemical modifications, alterations of intracellular macromolecular crowding, presence of suitable surfaces, absence of stabilizing ligands, impairment of intracellular quality control of protein folding, pH changes and others [217].
Most *in vitro* studies regarding the formation of amyloid fibers are based on lowering the pH, suggesting that mildly acidic pH (present for example in lysosomes) can induce rearrangement of the tetramer structure and dissociation into partially denatured monomeric amyloidogenic intermediates, that are then joined again to form amyloid fibrils [23]. However, it has been shown that normal TTR, as well as the variants V30M, L55P and the non-amyloidogenic T119M dissociate into monomeric species at physiological pH and ionic strength [30] and that the most unstable variants (V30M- and L55P-TTR) exist in a complex equilibrium between monomers, tetramers and aggregates of higher molecular weight [30].
Nowadays, it is thought that the critical step for amyloidogenesis is the destabilization of the structure and the formation of non-native intermediate species that have the ability to self-associate.

These intermediate species seem to play an important role both in the formation of amyloid fibrils as in the mechanisms of toxicity subjacent to amyloid disease.
Depending on conditions, during the process of amyloidogenesis different types of species can be formed: amorphous aggregates, soluble oligomers or amyloid fibrils [218-221], and the process rarely results in a homogeneous product. Usually, heterogeneous mixtures containing several species of aggregates (amyloid fibrils, amorphous aggregates

or soluble oligomers) are observed. Amorphous aggregates are formed faster than oligomers or fibrils and usually result from partially unfolded proteins that precipitate out of solution with no special conformational prerequisite to occur. Soluble oligomers are formed more slowly and remain in solution even after high-speed centrifugation, indicating that are not insoluble fibrillar or aggregated species. Fibrils are the slowest formed species and require special conditions to be formed.

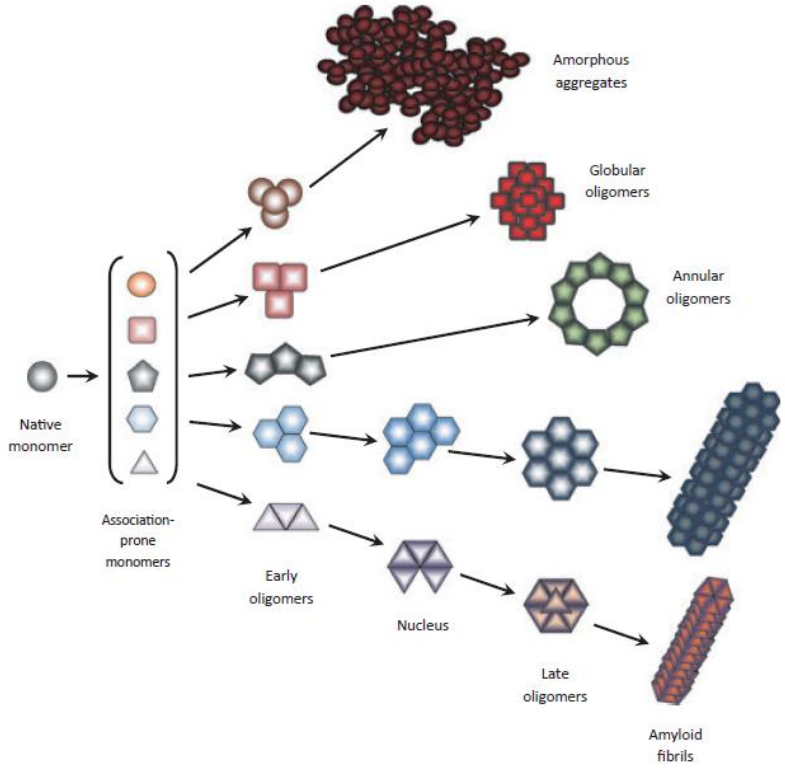


Figure 6 – A schematic representation of the protein self-association process, highlighting the three major products of the aggregation reaction: amorphous aggregates, soluble oligomers (spheroidal and annular) and amyloid fibrils. From Uversky 2010 [222].

The three main mechanisms proposed for TTR amyloidogenesis are: template-dependent, template-independent and proteolisis mechanisms [218].

**Template-dependent**

The aggregation of the protein results from a nucleation-polymerization process where addition of the monomers is thermodynamically unfavorable until a nuclear-core is formed [223]. Rather, the polymerization stage is thermodynamically favorable. The critic nuclear core is the oligomer with the minimum size cable of starting the elongation process [224]. In the case of TTR there is no evidence that amyloidogenesis proceeds this way [225].

**Template-independent**

Aggregation is a process that begins with the monomeric protein and ends with the formation of aggregates. In between, a number of steps occur, including the formation of different oligomers [222]. The model accepted for TTR is based on a conformational change, in which altered monomers are the building blocks for amyloid fibril formation [224, 226].

**Proteolysis hypothesis**

In some types of amyloidosis, the protein precursor is a subproduct of partial proteolysis (eg, reactive systemic amyloidosis (AA), immunoglobulin amyloidosis (AL), Alzheimer's disease (Aβ), gelsolin, cystatin C and apoA-I amyloidoses. In the case of TTR, C-terminal fragments are found, in addition to the full-length protein, in amyloid deposits of SSA and FAP patients [227]. However, these fragments are not always present, and their role is still controversial.

Recently, Pires *et al.* described the existence of distinct annular oligomeric intermediates formed during both the assembly and disassembly pathways of TTR protofibrils induced at acidic pH [228]. They suggested that annular oligomers undergo morphological transitions into spheroid oligomers and protofibrils, which can be reversed to annular oligomers at physiological pH.

In the TTR **assembly pathway**, aggregation was induced by acidification. Within the first hours, the sample was mostly populated by monomeric particles as a result of tetramer dissociation. After a few hours, annular oligomers with circular shape and octameric symmetry, as well as more compact spheroid oligomers were observed. The spacing of each subunit of the annular oligomers was consistent with the dimensions of a single wild-type TTR monomer. These annular oligomers may associate laterally and form spheroid oligomers and short protofibrils. After 7 days of incubation, only protofibrils and a small population of monomers/dimers remained. So, the annular oligomers seem to be a transient intermediate along the protofibrillogenesis pathway.

The **disassembly pathway** occurred when the TTR protofibrils that were formed in acidic conditions were exposed to physiological buffer. After 15 minutes exposed to physiological pH, there was dissociation of the protofibrils into annular oligomers, which were different from those observed during TTR assembly but may still serve as assembly blocks for another form of amyloid aggregation.

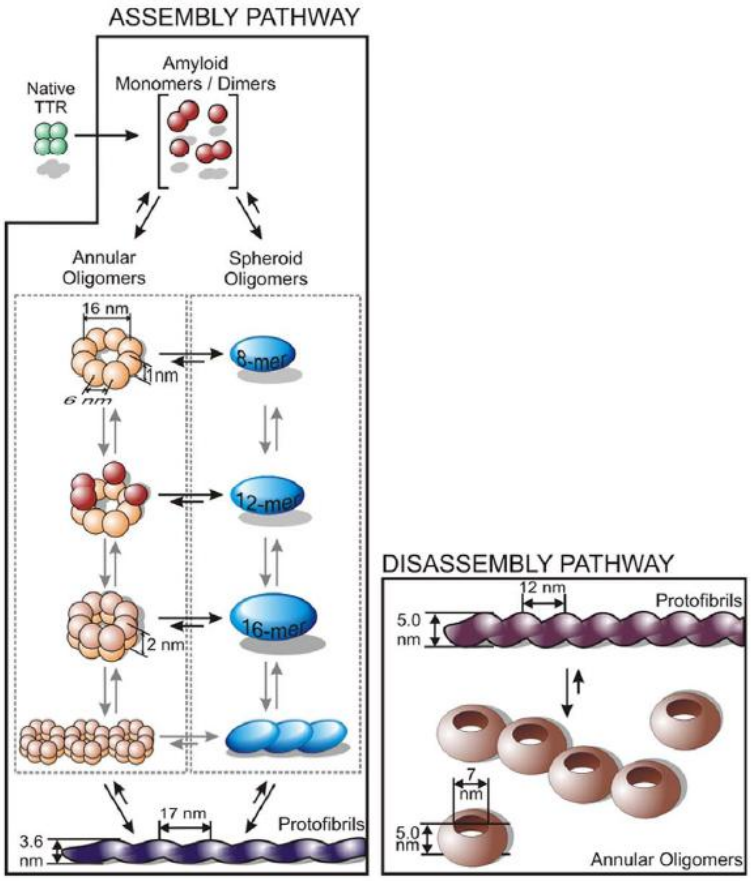


Figure 7 – Model of TTR protofibril assembly and disassembly. From Pires *et al.* [228].

So, in the protofibrillogenesis model proposed by Pires *et al.* the native TTR undergoes structural transitions upon acidification, and the tetramers dissociate into amyloidogenic monomers and, in smaller quantities, dimmers, from which annular oligomers with octameric symmetry assemble. A single annular oligomer may serve as a scaffold for the continuous addition of subunits up to the formation of an annular doublet. Spheroid oligomers coalesce in a dynamic equilibrium with the growing protofibrils which are abundant in the first week of incubation. Upon adjusting of pH to neutral, protofibril structure quickly reorganizes, and dissociation into an annular oligomeric species quite distinct from that seen in the assembly pathway proceeds in a time scale of minutes.

The annular oligomeric species are probably toxic, as it has been seen that oligomers containing up to eight TTR monomers, but not more, are cytotoxic [229].

### 5.4. Methods to induce the formation of oligomeric species *in vitro*

*In vitro* amyloidogenesis and formation of oligomeric species can be achieved by several methodologies, as long as the conditions that promote fibril formation are established. It is a difficult endeavour because variability exists between protein batchs and has been plagued by poor reproducibility of experiments both within and among laboratories [230]. Factors such as the purity of the protein sample, the ion-pairing agent used in the protein purification process, for example HCl and trifluoroacetic acid (TFA) buffers, the existence of pre-formed aggregates that can behave as fibril seeds and lead to accelerated amyloid formation, can contribute to this variability by affecting the kinetics of aggregation.
To promote fibril formation, the sample is incubated under a variety of different solution conditions (pH, temperature, and ionic strength). The most common methods are:

- incubating the protein with stirring at room temperature for 7 days [231]
- using low pH treatment of purified protein: diluting the protein in sodium acetate buffer at pH 3.6 and incubating at 37ºC [228]; diluting in acetate buffer, KCl and EDTA, pH varying from 4.8 to 6.0 [229, 232-233], followed by incubation at 37°C for a maximum of 7 days [229]
- incubating the protein for 48 h at room temperature in trifluoroethanol and sodium acetate, pH 5.5 [234].
- also, different concentrations of the protein and pH solutions, using either acetate or citrate buffer, may be used [235].

The choice of the method may depend on the protein involved or on the previous experience of the laboratory.

### 5.5. Cytotoxicity induced by oligomeric and pre-fibrilar TTR species

Mature amyloid fibrils were for long considered the responsible species for the pathogenic features of amyloid diseases, as they were the material commonly found in the pathological deposits. However, in later years, evidence has accumulated that the soluble oligomers and pre-fibrillar assemblies grown from the amyloidogenic proteins are the main or even the sole cytotoxic species [229, 236-239]. Most of these evidences come from cell culture-based experiments, as these soluble species have not been well characterized *in vivo*. Other circunstancial evidences, such as the fact that clinical manifestations of amyloidosis-related neurodegenerative diseases often precede detectable accumulation of the fibrillar protein aggregates, lend support to this theory. So, soluble oligomers are

considered very important players both in protein aggregation and in the related cytotoxicity.

The existence of early non-fibrillar TTR aggregates in pre-symptomatic FAP patients is supported by studies conducted by Sousa *et al.* [107]. The presence of these putatively cytotoxic aggregates is associated with the expression of markers of oxidative stress, and induction of apoptosis related molecules and pro-inflammatory cytokines [107], processes that may be common to the toxicity of prefibrillar amyloid protein aggregates in all amyloidotic diseases [234].
The mechanisms that mediate the cytotoxicity of oligomeric TTR aggregates are not yet fully understood. However, several pathways, including ER stress, oxidative stress and inflammation, have been implicated.

### 5.5.1. ER stress response in FAP

A connection between the endoplasmic reticulum (ER) stress response and FAP was demonstrated by Teixeira *et al.* [240]. Increased levels of the ER-resident chaperone BiP, a member of the heat-shock protein 70 family, were found both in human biopsies of FAP patients as well as in TTR transgenic mouse model [240]. It was also shown that extracellular TTR oligomers can induce BiP expression and activation of eIF2α in cell culture, involving the mobilization of $Ca^{2+}$ from the ER to the cytosol.
An N-glycosylated TTR fraction was identified in plasma of carriers of the V30M mutation, which was undetectable in plasma of normal individuals. This glycosylated TTR was secreted, escaping ER-associated degradation (ERAD) [241].

### 5.5.2. Disruption of biological membranes

Amyloidogenic species could be toxic by their capacity to interact and permeabilize the biological membranes, through mechanisms such as detergent, carpeting effects or pore formation [242]. Studies *in vitro* suggest that the TTR-mediated calcium permeability may be driven by binding of the misfolded protein directly to lipids of the plasma membrane, and that toxicity is correlated with increased membrane binding affinity, destabilisation of cell membrane fluidity and subsequent decrease in cell viability [243].

### 5.5.2. Apoptosis

Activation of caspase-3 was described in sciatic nerves of asymptomatic FAP individuals in which non-fibrillar TTR aggregates were observed [107], as well as in cells treated with pre-fibrilar aggregates [107]. The death receptor Fas as well as caspase-8 were found to

be up-regulated in tissues from animal models for FAP with non-fibrillar TTR deposition, as well as in salivary glands from FAP patients [244].

The TTRV30M protein itself may induce apoptosis and autophagy concomitant with the accumulation of reactive oxygen species (ROS) [245].

Nunes *et al.* found that TTRV30M decreases endothelial survival by inducing apoptosis [246]. In this study it was also shown that TTR could regulate angiogenesis, as endothelial cells seem to acquire different molecular identities when exposed to either wild-type TTR or TTRV30M proteins.

#### 5.5.3. Inflammation

The pro-inflammatory cytokines TNF-α, IL-1β and M-CSF (macrophage colony-stimulating factor) were found to be up-regulated in nerves from FAP patients [106-107]. IL-1β, in particular, was investigated as a possible therapeutic target in an FAP mouse model. Treatment with Anakinra, an IL-1 antagonist, prevented TTR extracellular deposition in sciatic nerve, protecting unmyelinated nerve fibers from aggregate-induced degeneration [247]. Moreover, Anakinra administration significantly inhibited apoptosis and nitrative stress, highlighting the relevance of the IL-1 signaling pathway in the pathophysiology of FAP. On the other hand, up-regulation of the anti-inflammatory cytokine IL-10 in axons and Schwann cells of FAP nerves, and its correlation with the presence of TTR amyloid deposits was also observed, suggesting a balance between pro- and anti-inflammatory mechanisms in FAP [248].

#### 5.5.4. Oxidative stress

Oxidative stress markers were found to be increased in axons of FAP patients even at pre-symptomatic stages of disease [107], as well as the levels of iNOS [244, 249] and of 3-NT (3-nitrotyrosine), a marker of protein nitration, in FAP nerves, suggesting that deposited TTR was subjected to nitration [106].

#### 5.5.5. The receptor for advanced glycation end products (RAGE)

It has been postulated that TTR aggregates can bind to the receptor for advanced glycation end-products (RAGE), which is highly expressed at the surface of different cell types in amyloid deposition sites, and activate extracellular signal-regulated kinase (ERK) cascades, leading to downstream increased nuclear transcription factor kB (NF-kB) activity, and activation of caspases [250-251]. The inflammatory cascades are consequently activated by NF-kB and in turn will promote NF-kB activation [252-253].

Several evidences suggest that both AGE and RAGE may have a common role in the progression of TTR amyloidosis, as amyloid proteins may directly bind to RAGE and

activate signaling pathways that result in cellular perturbations [251, 254]. However, conflicting results exist regarding the fact that the cytotoxic effects exerted by the oligomeric TTR may be triggered by activation of NF-kappaB or apoptosis [254].

### 5.6. EPO deficiency and TTRV30M aggregate toxicity: common mechanisms

As previously stated, TTR aggregates can bind to RAGE, which is highly expressed at the surface of different cell types in amyloid deposition sites, and activate ERK cascades, leading to downstream increased NF-kB activity, and activation of caspases. These mediators, as well as others involved in oxidative stress and apoptosis may influence EPO production. So, downstream events precipitated by the deposition of cytotoxic oligomeric species may be involved in the inhibition of EPO expression. Hypoxia Inducible Factor (HIF), GATA-2 and NF-kB are the main known regulators of EPO gene expression. HIF induces EPO expression under hypoxic conditions, while GATA-2 and NF-kB transcription factors suppress EPO promoter activity when up-regulated or activated by the pro-inflammatory cytokines IL-1 and TNF-α [168].

## 6. Renal Progenitor Cells

After acute renal damage, either ischemic or toxic, the kidney is able to regenerate and, in most cases, to completely recover its function and the integrity of the tubular epithelium. Recent studies have identified the renal cells that are responsible for this regenerative response. In man, a population of stem/progenitor cells, selectively localized at the urinary pole of the Bowman's capsule [255] was identified. It is characterized by the expression of the markers CD24 and CD133, as well as by expression of transcription factors characteristic of multipotent stem cells such as Oct-4 and BMI-1. The discovery of these progenitor cells in a region of the nephron which is contiguous with both the tubular structures and with podocytes [255], suggests that they represent common progenitors and can replace damaged cells in both structures. These cells are distributed along the Bowman's capsule and generate new podocytes, progressively migrating from the urinary pole to the vascular pole, where complete differentiation into podocyes take place [256-257]. Studies in mice suffering from glomerulosclerosis showed that these cells have the ability to integrate the mature structure, reducing proteinuria and improving the chronic renal damage [256]. When injected in mice with acute renal failure (SCID), the progenitors have the ability to regenerate different portions of the nephron and reduce renal damage, both morphological and functionally [255].

Cells expressing the surface markers CD133 and CD24, as well as vimentin, were also identified as tubular progenitors, close to the proximal tubular epithelium of the adult kidney [258]. Furthermore, in biopsies from patients with acute tubular necrosis, these CD133+ vimentin+ cells proliferate, so it may be assumed that they represent a population of progenitor cells [258]. In agreement with these observations, Sallustio and colleagues have confirmed the existence of CD133+CD24+ cells in the adult human kidney scattered along the tubular epithelium, both in proximal and distal tubular segments, and found that these cells have a gene expression profile not statistically different from that of the CD133+CD24+ cells of the glomerulus [259].

Studies from Angelotti and colleagues [260] went further and demonstrated that progenitor cells from the Bowman's capsule express a different marker VCAM1 (Vascular Cell Adhesion Molecule 1) or CD106, which is absent in the tubular stem cells, allowing to distinguish and separate them between CD133+CD24+CD106+ or CD133+CD24+CD106-. These CD106- cells are located in specific segments of the nephron: the proximal and

distal convoluted tubules. In turn, CD133+CD24+ cells were not identified at the level of the Henle's loop or of the collecting duct.

The progenitors from the Bowman's capsule exhibited a high proliferative potential and the ability to differentiate and acquire both the phenotype of podocytes and of tubular cells, while the tubular CD133+CD24+CD106- cells showed a much lower proliferative capacity and a phenotype already commited to tubular differentiation. Both populations, however, showed a capacity for greater resistance to toxic agents than already differentiated renal tubular cells. Moreover, both the glomerular CD133+CD24+CD106+ cells and tubular CD133+CD24+CD106- cells, if inoculated into mice with acute tubular injury, were integrated at tubular level and showed the ability to reduce kidney damage from both a morphologically and functionally point of view [260]. This suggests that both cell populations can potentially participate in the regeneration of cells in the adult human kidney.

The identification of the factors that regulate cell growth and differentiation properties of tubular progenitors opens important perspectives in terms of regenerative medicine. In the case of amyloidosis it is important to know if the misfolded protein influences the differentiation capacity of the renal progenitor cells. The development of potential therapeutic agents to prevent and treat tubular damage may depend on this knowledge.

## AIMS

# Aims

The results obtained over the last few years by our group have shown that erythropoietin (EPO) production is reduced in ATTRV30M amyloidosis (FAP) patients, leading to anemia at an early stage of the disease. Our previous studies have excluded a direct effect of either the presence of amyloid fibril deposits or of soluble circulating TTRV30M on EPO expression. As oligomeric TTR aggregates are now generally considered as the most cytotoxic species in ATTR amyloidosis, and suspected mediators of toxicity are likely to influence EPO expression, we decided to evaluate the effect of oligomeric TTRV30M aggregates on EPO expression.

Complementary, an assessement of oligomeric TTRV30M aggregate toxicity in the differentiation potential of the renal progenitor cells (RPC) CD133+CD24+ was carried out. These cells have the capacity to differentiate either as podocytes or as tubular structures, and are able to integrate *in vivo* different portions of the nephron, contributing to its regenerative potential.

So, in outline, the main objectives of this study are:

- To assess the cytotoxicity of oligomeric TTRV30M aggregates *in vitro*, using different target cell lines (Hep3B (human hepatocellular carcinoma), SH-SY5Y (human neuroblastoma cell line), HEK293T (human embryonic kidney cell line), and RPE (human retinal pigment epithelial cells)
- To evaluate the effect of oligomeric TTRV30M aggregates on EPO gene expression in Hep3B cells, and the role of known regulatory regions of the EPO gene.
- Establish a primary human EPO-producing cell line from retinal pigment epithelium (RPE) and evaluate the effect of TTR aggregates on these cells.
- To evaluate the effect of TTR aggregates on the viability and regenerative potential of renal progenitor cells (RPC) CD133+CD24+

# MATERIALS AND METHODS

### 1. Cell culture models and human renal biopsies

In this work we used both immortalized and primary cell lines in experimental models.

**Immortalized cell lines:**
Hep3B (human hepatocellular carcinoma)
SH-SY5Y (human neuroblastoma cell line)
HEK293T (human embryonic kidney 293 cell line)

**Primary cell lines:**
RPC (human renal progenitor cells) CD133+CD24+
RPE (human retinal pigment epithelial cells)

Hep3B cells were kindly offered by Dra. Sandra Alves (Department of Human Genetics, INSA, Portugal).

SH-SY5Y and HEK293T were kindly offered by Prof. Paola Romagnani's group (Excellence Centre for Research, Transfer and High Education (DENOTHE), University of Florence, Italy).

RPC cells were isolated from the glomeruli of adult human kidney, as described by Sagrinati *et al.* [255], by the collaborators of Prof. Paola Romagnani.

RPE cells were isolated from the choroid of an eye of an adult human cadaveric donor from Centro Hospitalar do Porto, in the setting of a project approved by the institutional ethics committee. The procedure for RPE isolation was adapted from Engelmann *et al.* [261] and Chung *et al.* [262]. Briefly, the RPE/choroid complex was placed in RPMI supplemented with 10% FBS, 10 µg/mL gentamicin, 0,5 mg/mL colagenase I and 0,5 mg/mL colagenase IV, was triturated with a bistoury and incubated for 4 hours at 37ºC. After incubation, the cells and media were collected and centrifuged for 5 minutes at 1400 rpm. The pelleted cells were washed with PBS, centrifuged and resuspended in RPMI supplemented with 10% fetal bovine serum (FBS) and 20 µg/mL gentamicin. The pigmented cells were counted using a neubauer chamber and plated at a density of 350.000 cells in a 25 $cm^2$ culture flask.

The RPC cells were maintained in EGM-MV (Cambrex Bio Science) supplemented with 10% FBS (Hyclone). All the other cell types were maintained in RPMI 1640 supplemented with GlutaMax (Invitrogen, CA, USA) and 10% FBS (Invitrogen). All cells were incubated at 37ºC in a 5% $CO_2$ atmosphere.

**Human renal biopsies**

In this study, 14 human formalin-fixed and paraffin embedded renal biopsies from renal cadaveric donors and ATTRV30M amyloidosis patients were used. Biopsies were provided by the Pathology Department of Santo António Hospital and informed consent for research use was obtained. Three renal biopsies from cadaveric donors whose kidney was successfully transplanted were selected as controls. No donor clinical data was available due to the anonymity rules imposed in organ collection procedures. Between 1995 and 2001, every FAP patient considered for liver transplantation was submitted to renal biopsy. Eleven biopsies from FAP patients were randomly selected, taking into consideration as inclusion criteria the absence of anemia, scarse deposition of amyloid in the kidney and normal renal function. FAP patients were on average 38 ±7 years old (29 – 56), with 4.5 ± 2.1 years (2 – 12) of symptomatic disease, and serum Hb of 13.2 ± 1.6 (10 – 16.2) g/dl.

## 2. Expression and purification of recombinant human TTRV30M

The *in vitro* studies conducted in this work evaluated the effect of TTR aggregates in the cellular models described in section 1 of this chapter. To do so, it was of crucial importance to obtain TTR preparations of very good quality, and free of any kind of contamination, such as endotoxin, to ensure that the effects observed with cells exposed to these preparations for several days were only due to their presence. To this end, we produced under stringent conditions recombinant human TTRV30M, using an *E. coli* expression system. As a control for some experiments we also used a sample of commercially available wild-type TTR (Sigma).

Recombinant Human TTRV30M was produced using a strain of chloramphenicol (cam) resistant BL21-RIL *E. coli* transformed with the made-to-order synthetic vector pJexpress401:34985–TTRV30M_v2_opt (pJT, DNA2.0 Inc.). Transformants were

selected for resistance to cam and kanamycin (kan), and the presence of the correct vector was confirmed.

Expression and purification of the recombinant protein was optimised. Briefly, a colony of BL21-RIL/TTRV30M clone (pJT) was grown overnight in 3 mL of LB medium with 50 µg/mL kan and 16 µg/mL cam at 37ºC and 220 rpm. The pre-culture was inoculated in 600 mL TB medium with the same antibiotics and grown for 4 h at 37ºC and 250 rpm. The expression of the protein was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), for 4 h at 30ºC and 250 rpm. The cells were collected by centrifugation and the pellet was stored at -20ºC.

To extract the protein, the pellet was dissolved in lysis buffer (50 mM Tris.HCl, pH 7.6, 200 mM NaCl, 10 mM imidazole) and treated with 100 µg/mL lysozyme, on ice for 30 min. The mixture was sonicated 3 x 30 s in pulse mode (0,5 s “on” and 0,5 s “off”) and centrifuged. The supernatant was filtered, first through a 0,45 µm membrane and then through a 0,2 µm membrane, and applied to a prepacked $Ni^{2+}$ sepharose high performance column (HisTrap HP, GE Heathcare) equilibrated in lysis buffer, an immobilized metal ion affinity chromatography (IMAC). After washing the column with 7 volumes of lysis buffer, a linear gradient of 10 - 400 mM imidazole was applied to elute the His-tagged protein. The elution peak fractions were pooled and their content verified by SDS-PAGE with Coomassie Blue or silver staining. The TTR containing fractions were dialysed overnight at 4ºC against $H_2O$, quantified by absorbance spectroscopy at 280 nm (Thermo Scientific NanoDropTM 1000 Spectrophotometer), using the extinction coefficient of wild-type TTR (wt-TTR), $\varepsilon 280 = 7.76 \times 10^4\ M^{-1}$ [263], and stored at -20ºC.

To remove the histidine tag, the chromatography product was cleaved with recombinant TEV protease, produced in our laboratory using the S219V TEV mutant, encoded in the pRK793 plasmid. This vector overproduces the catalytic domain of TEV protease in the form of an MBP fusion protein that cleaves itself *in vivo* to yield a TEV protease catalytic domain with an N-terminal His-tag and a C-terminal polyarginine tag, and was a kind gift from David Waugh (Addgene plasmid # 8827) [264]. It was expressed in BL21(DE3)-RIL cells, and TEV S219V purified by IMAC with a $Ni^{2+}$ sepharose high performance column, as described for TTRV30M. The cleavage reaction was performed at room temperature for at least 4 h in 50 mM Tris.HCl pH 7.6, 0.5 mM EDTA, 1 mM DTT, using a ratio of 1 mg of TEV for 10 mg of TTRV30M. Following digestion, the reaction was dialysed against 50 mM Tris.HCl pH 7.6, 200 mM NaCl and TEV protease was removed via its own poly histidine tag by IMAC. These TTRV30M fractions were pooled, concentrated and buffer exchanged with 50 mM Tris.HCl pH 7.6, by ultrafiltration, using a centrifugal filter device with a molecular weight cut off of 10 kDa.

An ion exchange chromatography was performed to further purify the recombinant TTRV30M. The protein was applied to a Q-Sepharose FF column equilibrated in 50 mM Tris.HCl pH 7.6. A linear gradient of 0 - 500 mM NaCl was applied to elute the TTR.
Finally, the TTR containing fractions were concentrated by ultrafiltration and applied to a gel filtration Sephacryl S-200 column equilibrated with 10 mM phosphate buffer pH 7, 100 mM KCl and 1 mM EDTA. The appropriate fractions were pooled and concentrated by ultrafiltration.
Purified soluble TTRV30M was detoxified using ActiClean Etox (Sterogene). The protein was eluted with PBS (Invitrogen), concentrated to a final concentration of 2 mg/mL and filter sterilized by passing through a 2 µm seringe filter. Final concentration was determined by measuring the absorvance with a NanoDrop 1000 spectrophotometer, using the extinction coefficient of wt-TTR, ε280 = 7.76 x $10^4$ $M^{-1}$. A limulus amebocyte lysate-based assay (E-Toxate Test, Sigma, MO, USA) was used to confirm that the protein was endotoxin-free.

## 3. Preparation and characterization of TTR amyloidogenic aggregates

In the literature, several methods have been described for the production of oligomeric aggregates. Fibrillogenesis depends on various conditions such as temperature, protein concentration, ionic strength, agitation and pH [265]. The misfolding of the protein is an essential step. In the case of TTR, the most common method is based on the acidification of the protein solution to mildly acidic pH (~4-5) to mimic the lysosomal milieu, as it may be sufficient to initiate the assembly of TTR amyloid fibrils [266]. Complete unfolding of the protein into monomers with HCl pH 2.0 followed by refoldig with NaCl has also been described [267]. Another method of achieving oligomerization is by aging the protein, with or without stirring, at physiological pH [268].
In this work, three different conditions were tested to induce aggregation: moderate pH, unfolding and refolding (HCl and NaCl) and aging. Regardless the method used, we found that the fibrillation process is very complex and not always reproducible, whatever the method used.

### 3.1. TTR aggregation at mild pH (4.0-5.5)

Aggregation was induced by lowering pH to 4.0 [269], using a sodium acetate solution, or to pH 5.5 using trifluoroethanol and sodium acetate, methods adapted from Reixach *et al.* [229] and Bucciantini *et al.* [238], respectively. Different aggregation times were tested.

Briefly, 1,5 mg/mL TTRV30M was incubated with either 100 mM sodium acetate buffer, pH 4.0, as with a mixture of 10% trifluoroethanol and 50 mM sodium acetate pH 5.5, at room temperature and at 37ºC. At different time points, the amyloid formation process was followed by spectrofluorimetry with Thioflavin T (ThT).

### 3.2. TTR aggregation by unfolding with HCl and refolding with NaCl

A 30 µM TTRV30M protein solution was dialysed for 96 h against 10 mM HCl (pH 2.0) to unfold the tetramer into monomers and the aggregation was induced with NaCl, as described by Lindgren *et al.* [267]. Final concentrations of 100 mM and 50 mM of NaCl, well as different incubation times, were tested. At different time points, the formation of amyloid was evaluated by spectrofluorimetry with ThT and cross-linking assays.

### 3.3. TTR aggregation at physiological pH followed by magnetic stirring

A solution of 36 µM TTR V30M (2 mg/mL) in PBS pH 7.4 (Invitrogen) was filtered through 0,2 µm Anotop syringe filters (Whatman, Kent, UK) and incubated at 37ºC for 72 hours, followed by vigorous stirring for 5 minutes, using a magnetic stirring bar. The size of the molecular species in solution was evaluated by Dynamic Light Scattering (DLS). Oligomers from wild-type TTR (wt-TTR) were prepared by incubating a commercial protein (Sigma-Aldrich) in PBS, pH 7.4 for 96 hours at 37ºC, followed by vigorous stirring for 25 minutes.

## 4. Characterization of TTR amyloidogenic aggregates

The formation of TTR oligomeric aggregates was evaluated by the following methods:

### 4.1. Thioflavin T assays

ThT assay was adapted from the method described by Nilsson [230]. Briefly, a 12,5 mM ThT was prepared in 50 mM Tris pH 7.8 and filter sterilized. Aliquots from the TTR aggregation reaction at different time points were mixed at a final concentration of 0,02 mg/mL with the assay buffer at 25 µM ThT. The fluorescence intensity for each sample was measured by excitation at 440 nm and emission 482 nm, using a Spectramax Gemini XS Reader. An aliquot of untreated protein solution and of Tris buffer were also added to the ThT assay buffer and intensity measured, to serve as control sample and negative control, respectively. A measured intensity above the control sample is indicative of the presence of amyloid fibrils.

### 4.2. Chemical cross-linking assays

Chemical cross-linking, followed by SDS-PAGE and silver staining, was performed to visualize the formation of TTR oligomers of different size according to the aggregation time [229]. Briefly, aliquots from the TTR aggregation reaction at different time points were mixed at a final concentration of 0,4 mg/mL with a 2.5% solution of glutaraldehyde, and incubated for 5 min at 37ºC. The reaction was terminated by addition of 1 M Tris-HCl, pH 8.0 to a final concentration of 100 mM. The samples were evaluated by SDS-PAGE and silver staining.

### 4.3. Dynamic light scattering (DLS)

DLS measurements were performed at 25ºC in a Malvern Zetasizer Nano ZS (Malvern, Worcestershire, UK) [268] to characterize tetrameric (time zero) and oligomeric TTR V30M preparations. Each sample was measured three times; average distributions are presented.

## 5. Cell toxicity, apoptosis and caspases 3/7 assays

It has been postulated that oligomeric species, rather than mature fibrils, induce citotoxicity *in vitro*. Mainly neuronal cells (neuroblastoma) [106, 229, 250, 270-272], and primary human umbilical vein endothelial cells (HUVECs) [246] have been used in these cytotoxicity studies. In this work we used both immortalized and primary cell lines to evaluate the cytotoxic effect of the oligomeric TTR aggregates in these cells in terms of cell viability, apoptosis and activity of caspases 3/7. Three distinct aggregation methods were tested in order to establish the best conditions.

### 5.1. MTT and MTS cell viability assays

Cells were plated into 96-well plates in complete cell medium (EGM-MV supplemented with 10% FBS for RPC or RPMI 1640 supplemented with GlutaMax and 10% FBS for all the other cell types) at a density of 3 x $10^3$ cells per well and incubated overnight at 37ºC and 5% $CO_2$. The next day the medium was removed and 100 µL of fresh medium with 0.5% FBS and 2 µM of each protein preparation added. The non-radioactive cell proliferation assays MTT (Promega, Wisconsin, USA) or MTS (Promega, Wisconsin, USA) were performed after 24, 48 and 72h of incubation, according to the manufacturer's instructions. The absorvance was read in a microplate reader at 580 nm for the MTT assay and 490 nm for the MTS. The result was expressed as “percentage of living cells”

relative to that seen in control using the expression 100 × (ODsample - ODblank)/(ODcontrol - ODblank). The ODblank was established from the average of the wells containing only medium. Average values and SD were calculated from triplicate determinations.

### 5.2. Annexin V apoptosis assays

Renal progenitor cells (RPC) were plated at a density of 100.000 cells/well in a 6-well plate in EGM-MV with 20% FBS and incubated overnight at 37ºC and 5% CO2. The cell medium was changed to EBM without serum and the cells incubated for 7 h. Protein preparations (2 µM) diluted in EBM with 0.5% FBS were added to each well and the cells incubated for 48 and 72 h. The cells were detached, washed with PBS and resuspended in FACS Buffer (10 mM HEPES, 140 mM NaCl, 2 mM $CaCl_2$, pH 7.4) at 1 x $10^6$ cells/mL. Cells were stained with Annexin V-APC (eBioscience BMS306APC/100) and propidium iodide (PI) (Invitrogen) at a final concentration of 5 µg/mL and incubated for 15 min at room temperature, then kept on ice. Cell were analysed by flow cytometry in a BD LSRII flow cytometer (BD Biosciences).

### 5.3. Caspases 3/7 assays

SH-SY5Y, Hep3B and RPE cells were plated as for the MTS assay. 2 µM of each protein preparation, diluted in RPMI 1640 with 0,5% FBS was added to the respective wells. After 24 h of incubation at 37°C, the Caspase-Glo 3/7 Assay (Promega, Wisconsin, USA) was performed to determine the activity of caspases 3/7, according to the manufacturer's instructions. Luminescence was read in a Vitor3 spectrophotometer (Perkin Elmer) and the activity of caspases 3/7 was expressed in luminescence units. Average values and SD were calculated from triplicate determinations.

## 6. Influence of TTR oligomeric aggregates in the cell cycle and differentiation capacity of renal progenitor cells

### 6.1. Cell Cycle analysis

RPC were plated at 1 x $10^5$ cells/well in a 6-well plate the day before the experiment. Solutions of 2 µM TTRV30M diluted in EBM with 0,5% FBS were added to each well. The cells incubated for 48 and 72 h with the stimuli, then were detached, washed and resuspended in 100 µL 50% FBS in PBS, then fixed at 4ºC for 1 hour with 300 µL cold 70% ethanol added dropwise, washed, stained with Propidium Iodide (50 µg/mL) in

presence of RNAse 100 µg/mL and analyzed by flow cytometry in a BD LSRII flow cytometer (BD Biosciences).

### 6.2. Differentiation of RPC into podocytes

Following Ronconi *et al.* [256], RPC were plated at 8 x $10^4$ cells/well in a 6-well plate with the differentiation medium VRAD: DMEM-F12 supplemented with 0,5% FBS, 100 nM vitamin D3, and 100 µM ATRA. For the non differentiation control, the medium used was EBM with 0,5% FBS. The oligomeric protein was diluted in the correspondent media, at a final concentration of 2 µM. Differentiation was induced for 24 and 48 h. The cells were detached, counted and centrifuged. The pelleted cells were used for RNA extraction, RT-PCR and real time PCR. Differentiation was evaluated by quantification of nephrin and glyceraldehyde-3-phosphate dehydrogenase gene expression in taqman real-time PCR assays, using standard curves generated with serial dilutions of the same positive sample.

## 7. Influence of TTR oligomeric aggregates on the expression of the erythropoietin gene in Hep3B and RPE cells

The human hepatoma Hep3B cell line expresses erythropoietin (EPO) both in a constitutive and an inducible oxygen-dependent manner [273]. Due to the lack of an adequate renal EPO-producing cell line, we used a human hepatoma Hep3B culture model to evaluate the influence of TTR oligomeric aggregates directly on the expression of the erythropoietin gene, both in normoxia and in simulated hypoxia, using the panhydroxylase inhibitor dimethyloxalylglycine (DMOG). Retinal pigment epithelial (RPE) cells are known to be local EPO producers, so primary cell cultures of RPE were also used.

Hep3B and RPE cells were plated into 12-well plates in RPMI supplemented with 10% FBS at a density of 8 x $10^4$ cells per well. The next day the medium was removed and each TTR preparation, diluted in RPMI 1640 supplemented with 0,5% FBS, was added in quadruplicate at a final concentration of 2 µM. After 16 h of incubation at 37ºC, the panhydroxylase inhibitor dimethyloxalylglycine (DMOG) (Sigma) was added to 2 wells of each preparation to a final concentration of 200 µM, to mimic hypoxia, and cells incubated for another 8 h.

Total RNA was extracted using Trizol Reagent (Invitrogen), according to manufacturer's instructions. RNA was quantified by measuring the absorbance at 260 nm, and was

treated with 0,2 units of RNase-free DNase I (Promega) per µg of RNA, was further purified by ethanol precipitation and resuspended in RNase-free water. First-strand cDNA was synthesized from 1 µg of RNA using Superscript III DNA polymerase (Invitrogen). Real-time PCR quantification was performed in a Corbett Rotor-Gene 6000 (Qiagen, Hilden, Germany) using Taqman Gene Expression Master Mix (Life Technologies, CA, USA). A fragment of the EPO gene was amplified using Lux Primers (Invitrogen) [274] with the following sequences: forward primer 596F (5'-CTGGGAGCCCAGAAGGAAG-3') and reverse primer 648RL (5'-CACTCCAGCAGTGATTGTTCGGAGTG-3', internally labeled with FAM). The cycling conditions were: one cycle at 50ºC for 18 min; one cycle at 95ºC for 10 min; 45 cycles of 95ºC for 20 s, 58ºC for 40 s, 72ºC for 40 s. The specificity of the amplification was confirmed by sequencing the PCR amplicons and by melting curve analysis. Results were normalized to endogenous TATA-binding protein mRNA (TBP), using the probe/primer set Hs00427620_m1 (Life Technologies) for taqman gene expression assays.

## 8. Influence of TTR oligomeric aggregates on the activity of the EPO promoter

### 8.1. Cloning of the erythropoietin promoter

A DNA fragment of 1848 bp, containing the base pairs -1772 to 76 relative to the EPO transcription site which includes the recognition sites for the transcription factors GATA-2 and NF-kB, was PCR amplified from genomic DNA of a normal individual, that gave informed consent for research use, using the following primers: forward, 5'-GCCAGATCCCGCAATACTCAC-3'; reverse, 5'-CTGGAGGAGAGGGCGGCTGTC-3'. The PCR product (figure 8A) was purified using a commercial kit (Promega) and sequenced using the "BigDye Terminator v3.1 Cycle Sequencing Kit" (Applied Biosystems). The fragment of the EPO promoter was first subcloned into the pDrive Cloning Vector (Qiagen) (shown on figure 8B), and then transferred into the *MluI-XhoI* restriction site of the pGL3-basic vector (Promega). The insert was verified by restriction enzyme digestion with *MluI* and *XhoI*, and bidirectional Sanger sequencing. A scheme of the constructed vector Epo-Prom-pGL3 is depicted on figure 9.

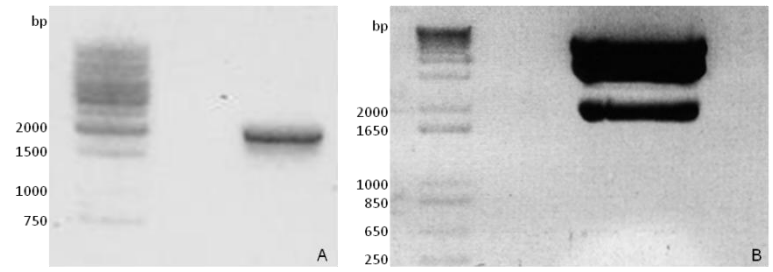


Figure 8 – Electrophoresis in 1,5% agarose gel, stained with ethidium bromide 1 µg/mL of the pcr product of the EPO Promoter, comprising base pairs -1772 to 76 relative to the EPO transcription site (A); insert of the EPO Promoter pcr product cloned into the pDrive Cloning Vector and verify by cutting the plasmid with the restriction enzyme *EcoRI* (B).

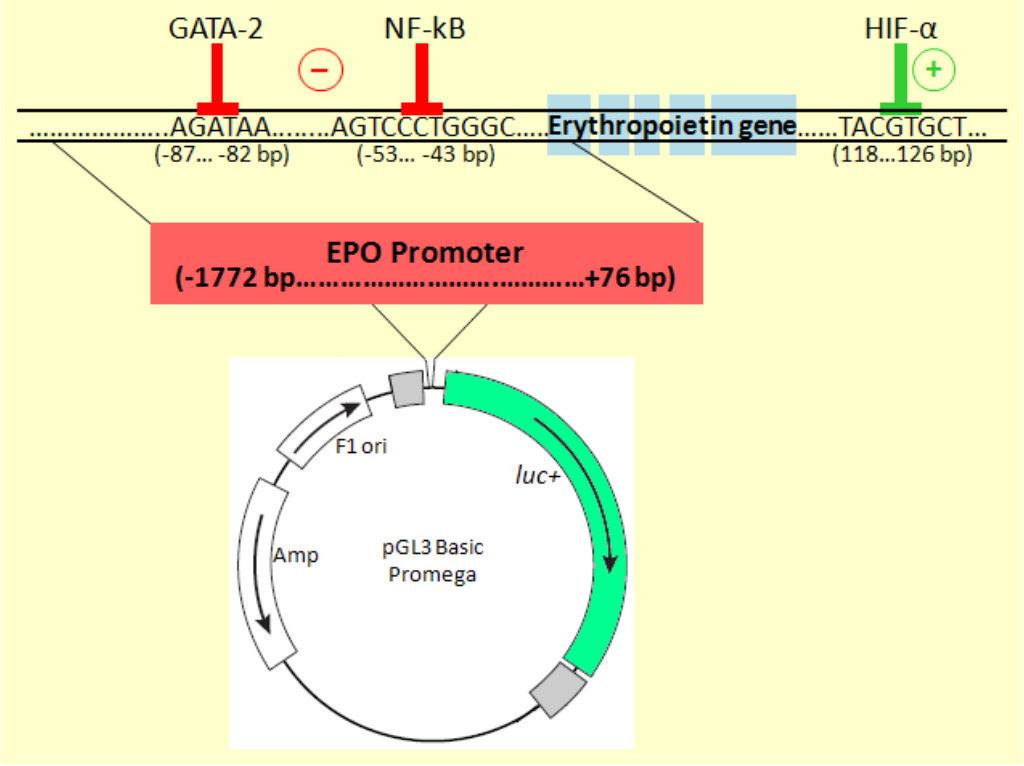


Figure 9 – Scheme of the reporter vector Epo-Prom-pGL3 constructed for the luciferase assays. The vector contains a fragment of the EPO promoter spanning the sequence binding sites for GATA-2 and NF-kB, which are known to negatively regulate EPO gene expression in normoxia.

### 8.2. Transfection with Epo-Prom-pGL3

Hep3B cells were plated into 24-well plates in complete cell medium at a density of 9 x $10^4$ cells per well on the day before the experiment. To evaluate the influence of TTR V30M on the activity of EPO promoter, Hep3B cells were cotransfected with 7 ng of the control Renilla luciferase plasmid pTK-RL (Promega) and 350 ng of the Epo-Prom-pGL3 plasmid. Transfections were done with Lipofectamine 2000 (Invitrogen), using a ratio of 1:3 of DNA (µg) to lipofectamine (µL) respectively. After 18 hours, the medium was removed and each TTR preparation was added, at a final concentration of 2 µM. Cells were incubated for 24 h and luciferase activities were measured with the Dual-Luciferase Assay Kit (Promega), using a Victor 3 multilabel reader (PerkinElmer). Fold induction was calculated using Renilla luciferase control normalized values.

### 8.3. Co-transfection with Epo-Prom-pGL3 and pCG-ATF3

Hep3B cells were plated into 24-well plates in complete cell medium at a density of 9 x $10^4$ cells per well on the day before the experiment. To evaluate the possible influence of the activating transcription factor ATF3 on the activity of EPO promoter in Hep3B cells exposed to TTRV30M, cells were cotransfected with 14 ng of the control Renilla luciferase plasmid pTK-RL (Promega), 350 ng of the Epo-Prom-pGL3 plasmid and 350 ng of pCG-ATF3 (kindly offered by professor Tsonwin Hai from the Ohio State University). Transfections, incubations with 2 µM of TTR preparations and measurements of luciferase activities were done as described in the previous section. Fold induction was calculated using Renilla luciferase control normalized values.

### 8.4. Immunofluorescence for NF-kB and GATA-2 on Hep3B cells

Hep3B cells were plated on 4-well chamber slides (Lab-Tek) at a density of 7,5x$10^3$ cells/well and incubated overnight in complete cell media. Solutions of oligomeric or tetrameric TTRV30M, 2 µM in RPMI with 0,5% FBS, were added to each well and the cells incubated for 24 hours. The cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with cold methanol at -20ºC for 10 min. Cells were washed with PBS between each incubation. Blocking was performed with 5% BSA in PBS for 1 hour followed by incubation with the primary antibody diluted in 1% BSA for 3 hours at room temperature. The antibodies used were rabbit anti-human NF-kB p65 polyclonal antibody (ab7970, Abcam) diluted 1:400 and rabbit anti-human GATA-2 polyclonal antibody (H-116, Santa Cruz Biotechnology) diluted 1:600. Cells were washed with PBS and incubated for 1 hour with the secondary antibody (A11008, Alexa Fluor 488 goat anti-rabbit IgG, Life Technologies) diluted 1:600 in 1% BSA. Slides were mounted in Fluoroshield with DAPI (Sigma) and analyzed using a fluorescence microscope (Leica).

### 8.5. Immunohistochemistry for NF-kB and GATA-2 on FAP renal biopsies

Immunohistochemistry (IHC) was performed on 4 μm thick formalin-fixed paraffin-embedded sections using standard methods. Treatment with 0.01 M Sodium Citrate Buffer (pH 6.0) was performed to enhance antigen retrieval. Blocking was performed with 5% BSA in PBS for 1 hour at room temperature, followed by incubation with the antibodies diluted in 1% BSA in PBS for 3 hours at room temperature. The antibodies used were rabbit anti-human NF-kB p65 polyclonal antibody (anti-NF-kB, Abcam) diluted 1:400, and rabbit anti-human GATA-2 polyclonal antibody (Santa Cruz) diluted 1:600. Detection was carried out with Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako).

### 9. Statistical analysis

Results of replicated experiments were expressed as mean±SD. Means were compared using the Student's t-test. P values $<0.05$ were considered statistically significant.

# RESULTS AND DISCUSSION

### 1. Production and evaluation of TTRV30M aggregates

Recombinant TTRV30M protein was obtained using an *E. coli* expression system. The synthetic vector pJexpress401:34985–TTRV30M_v2_opt (pJT, DNA2.0 Inc.) codes for a fusion protein containing the human TTR with the Met30 mutation and a poly-histidine tag that enables its purification by IMAC, and that can be subsequently removed.

The recombinant protein accumulates inside the cell, in inclusion bodies, a process that was minimized by decreasing the incubation temperature to 30ºC at the time of protein expression induction with IPTG. After lysing the cells, the protein was released to the medium in soluble form and the supernatant was directly applied to the $Ni^{2+}$ sepharose column. A linear gradient of imidazole was run through the column, and the TTRV30M protein was eluted with a concentration of imidazole of approximately 300 mM, in an almost pure form (figure 10). On average we obtained 40 mg of TTRV30M-HIS tagged / L of cell culture, which was equivalent to approximately 2 mg of TTRV30M-HIS tagged per gram of bacterial pellet.

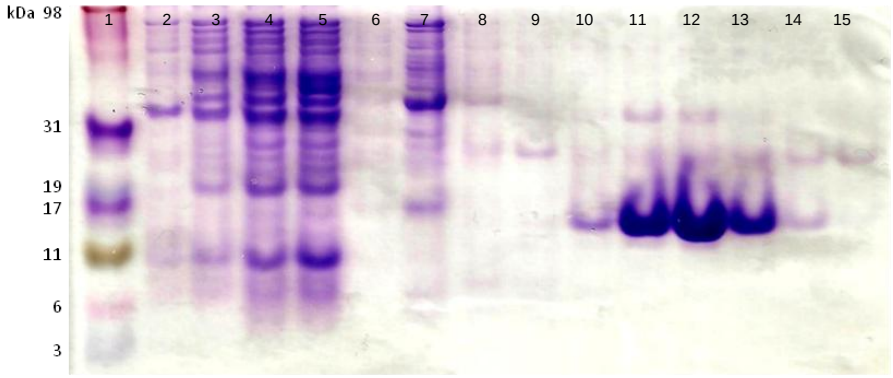


Figure 10 – IMAC elution profile of the rh-TTRV30M; lane 1: MW ladder; lanes 2-5: flowthrough; lanes 6-15: linear gradient of imidazole; lanes 10-14: fractions containing TTRV30M.

After TEV protease treatment, the histidine tags and protease were removed in the end by passing the reaction mixture through a second $Ni^{2+}$ sepharose column, to obtain a relatively pure cleaved TTRV30M (figure 11).

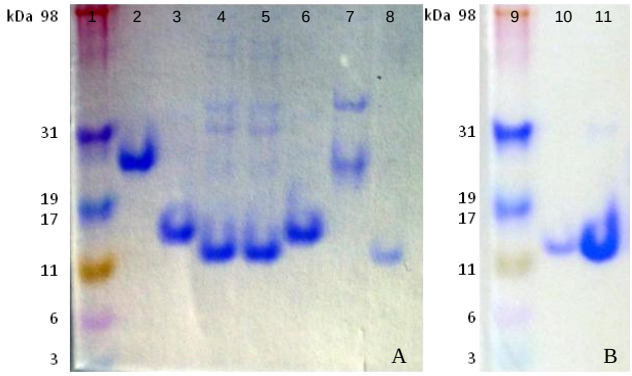


Figure 11 – Proteolysis of his-tagged TTRV30M by TEV protease (A) and cleaved TTRV30M after IMAC purification and concentration by ultrafiltration (B); lanes 1 and 9: MW ladder; lane 2: TEV793; lanes 3 and 6: his-tagged TTRV30M; lanes 4 and 5: completed proteolysis reaction, before IMAC purification; lane 7: peptides and TEV that remained attached to the column, eluted with 400 mM imidazole; lanes 8 and 10: purified TTRV30M; lane 11: TTRV30M purified and concentrated by ultrafiltration.

To further increase the purity of the protein, two more chromatographic steps were performed: an ion exchange chromatography, using Q-Sepharose FF, followed by a polishing gel filtration chromatography, to eliminate possible protein aggregates that may be formed during the purification process. The last step was the chromatographic removal of LPS. A limulus amebocyte lysate-based assay (E-Toxate Test, Sigma, MO, USA) was used to confirm that the final protein batch was endotoxin-free.

Due to the extensive purification process, involving two IMAC chromatographies, one ion exchange chromatography, a gel filtration chromatography and finally detoxification, the yield of purified TTRV30M was approximately 45% of the initial TTRV30M-HIS tagged pool. We obtained an average of 18 mg TTRV30M / L of cell culture. Although the yield is relatively low, we obtained a highly pure protein to be used on the cell culture assays.

## 2. Evaluation of TTRV30M amyloidogenic aggregates

Throughout the work, three different methods were used to produce the TTRV30M aggregates, according to the literature: acidification of the protein solution to mildly acidic pH (~ 4-5) [266], complete unfolding of the protein into monomers with HCl pH 2.0 followed by refoldig with NaCl [267], and aging the protein at 37ºC in physiological pH, followed by 5 minutes magnetic stirring, a method that we adapted from the procedure described by Ferreira *et al.* [268]. The reason for testing different methods was the poor reproducibilitty of the oligomerization process. Regardless the method used, we found that the fibrillation process to be very complex and difficult to reproduce.

Fibrillation of TTR seems to proceed by disruption of the tetramer into monomers, which undergo a conformational change, and are the building blocks for the formation of fibrils [224, 275-278]. Other models implicate the dimer [279] or the oligomers [280] as the building blocks. Irrespective of the model proposed, there are common evidences regarding which species are responsible for the cytotoxicity induced in the tissues. The intermediate species or small soluble oligomeric aggregates that are formed during the fibrillation process are toxic to cells in a free radical dependent manner [271], while the amyloid fibrils themselves are not toxic to the cells. Amyloid fibrils have been proposed to be protective, by storing the misfolded proteins in non-toxic fibrils and thereby removing toxic protofilaments from circulation [271].

To evaluate the formation of amyloidogenic TTRV30M aggregates, Thioflavin T (ThT), chemical crosslinking or dynamic light scattering (DLS) were used.

Upon addition of thioflavine T to suspensions of TTRV30M incubated with either 100 mM Acetate Buffer, pH 4.0, with a mixture of 10% trifluoroethanol (TFE) and 50 mM sodium acetate pH 5.5, or with HCl pH 2.0 and 100 mM NaCl, the fluorescence intensity increases (figure 12), indicating formation of amyloidogenic species.

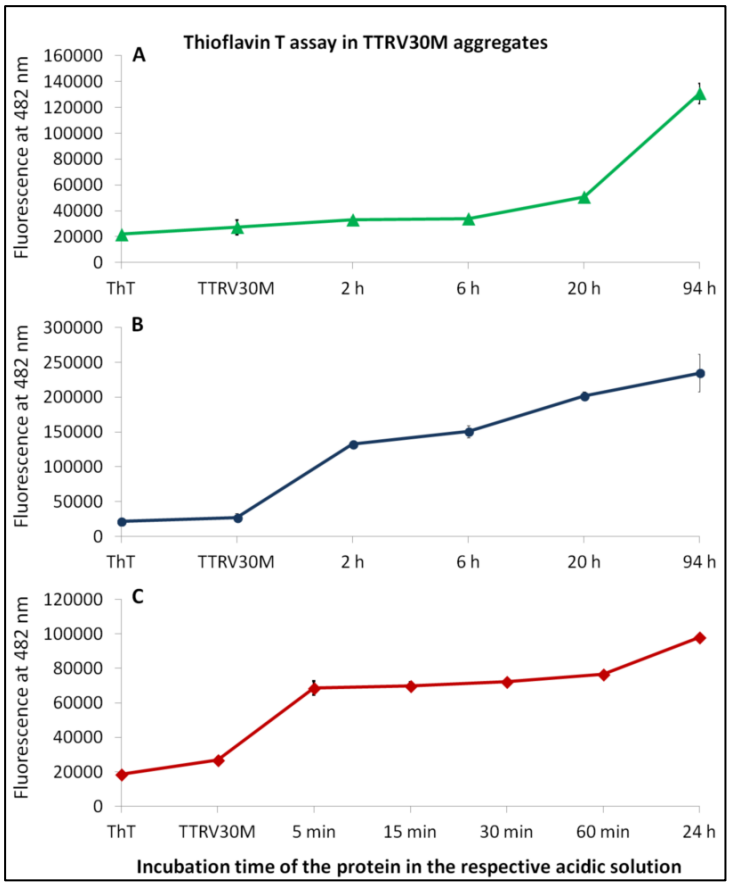


Figure 12 – Time course of TTRV30M aggregation followed by ThT fluorescence, induced by acetate buffer pH 4.0 (A), acetate buffer pH 5.5 and 10% TFE (B) or HCl pH 2.0 followed by 100 mM NaCl (C).

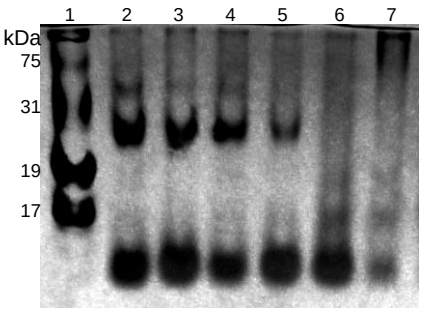


Figure 13 – Cross-linking with glutaraldehyde followed by SDS-PAGE with silver staining showing TTRV30M tetramer dissociation with HCl at pH 6 (2), 5 (3), 4 (4), 3 (5), 2 (6) and formation of aggregates at pH 2 and incubation for 5 minutes with 100 mM NaCl (7).

In the case of acetate buffer at pH 4, the intensity of fluorescence increased slowly over time (figure 12A). On the other hand, for the TTRV30M incubated with either a mixture of TFE and sodium acetate pH 5.5 (figure 12B) or with HCl and NaCl (figure 12C), the aggregation reaction started immediately. Figure 13 shows dissociation of the TTRV30M tetramer into monomers as a function of pH and the rapid formation of larger species upon addiction of NaCl. These methods, based on acidification of the protein solution to induce amyloid formation are described in the literature and used to produce cytotoxic aggregates.

We tested these aggregated TTRV30M preparations on cell culture assays. Despite positive ThT results, we had no significant cytotoxic effect with the solutions prepared with acetate buffer pH 4.0 or with acetate buffer pH 5.5 and TFE. On the other hand, we obtained a moderate cytotoxic effect with the protein solution prepared by acidification

with HCl and induction of aggregation with NaCl for 5 minutes. Interestingly, the solutions prepared with the acetate buffers and TFE usually precipitated immediately, indicating formation of large amorphous aggregates. On the other hand, the solution of TTRV30M in HCl was completely transparent, even after the addiction of NaCl, probably due to the formation of soluble oligomers. Although the ThT assays done did indicate the presence of amyloid-like species, no correlation with the cytotoxicity induced by the protein solutions was apparent.

As the aim of our work was to use the TTRV30M oligomeric preparations on cell culture assays, and to more accurately approximate the physiological environment, we also tested the method based on aging the protein solution while maintaining the physiological pH, as described by Ferreira *et al* [268]. We made a slight change, by aging the TTRV30M solution at 37ºC under stagnant conditions and then stirring vigorously for only 5 minutes.

DLS was used to evaluate the size of the TTRV30M species in solution. Soluble tetrameric TTRV30M, diluted in PBS at pH 7.4, at time 0 showed a single peak at approximately 7 nm (figure 14A). To induce the formation of oligomers, TTRV30M was incubated for 72 hours at 37ºC, followed by 5 minutes of vigorous stirring. At the end of the 72 h incubation, the presence of oligomeric aggregates with 150 nm was noted (figure 14B), that progressed very rapidly to larger 300 nm aggregates after vigorous stirring, representing then the majority of the TTR preparation (figure 14C). A preparation stirred for 7 days showed a predominance of species of approximately 600 nm (figure 14D). However, this solution did not induce cytotoxicity, unlike the solutions containing species of 150 and 300 nm.

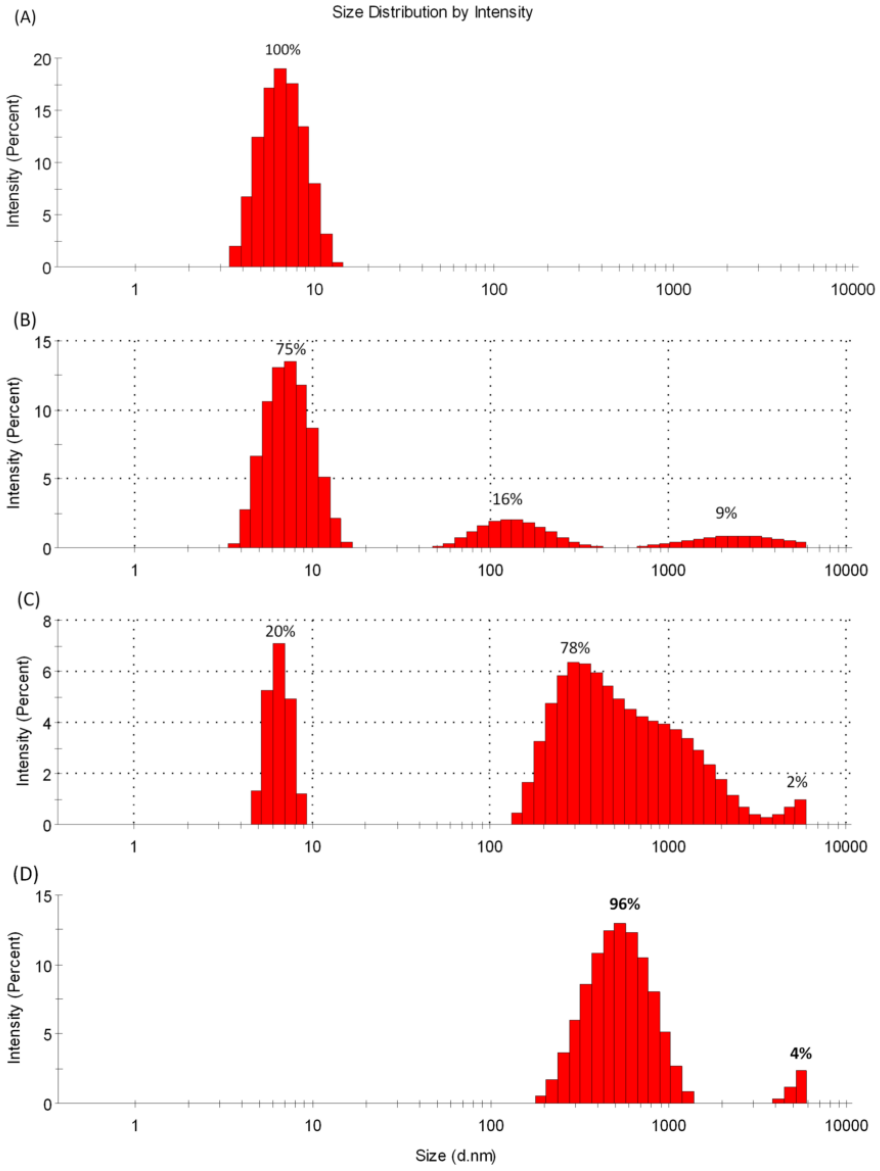


Figure 14 - DLS analysis of TTR V30M aggregation for 0 (A), 3 days at 37º C under stagnant conditions (B), 3 days at 37º C under stagnant conditions followed by 5 minutes of magnetic stirring (C), 7 days of magnetic stirring (D). Preparation represented in (C) has a high percentage of small oligomeric species (78%) so was the one used for the cell culture assays.

TTRV30M is a tetrameric protein with an extensive β-sheet conformation, prone to dissociation into intermediate molecular species during amyloid fibrillogenesis. Pires *et al*. reported that distinct annular oligomeric intermediates formed along both the assembly and disassembly pathways of TTR, that undergo morphological transitions into spheroid oligomers and protofibrils [228]. We induced protein fibrillation *in vitro* by aging a solution of TTRV30M at physiological pH and temperature, which is evidence for the natural

instability of the quaternary structure of the protein and its tendency to aggregate and form amyloidogenic fibers. We saw that after 72 hours of incubation under stagnant conditions, species of molecular size corresponding to early oligomers are already in solution, albeit in small amounts relative to the predominant tetrameric form. Stirring this solution for 5 minutes was sufficient to trigger the rapid formation of larger species.

TTRV30M preparations obtained by aging at physiological pH followed by magnetic stirring for 5 minutes (figure 14C) and preparations obtained by dissociation of the tetramer into monomers at pH 2.0 followed by refolding with NaCl (figure 12C) were the final preparations of oligomeric TTRV30M used for the cell culture assays, as we saw that these were the most cytotoxic species.

### 3. TTRV30M oligomeric aggregates compromise cell viability of both immortalized SH-SY5Y, Hep3B and HEK293T cell lines, as well as of primary RPE and RPC cells

The immortalized cell lines Hep3B, SH-SY5Y and HEK293T, the primary RPE cells and the renal progenitor cells RPC were exposed to TTR preparations in order to evaluate cell viability. Oligomeric preparations were prepared by unfolding with HCl and refolding with NaCl, or by aging the protein solution at pH 7.4 and 37ºC followed by 5 minutes of stirring. Hep3B and SH-SY5Y were exposed to oligomers produced by both methods. HEK293T and RPC were exposed only to oligomers produced by unfolding and refolding. RPE cells were exposed only to oligomers produced by aging the TTRV30M at physiological pH and temperature.

#### 3.1. Cell viability assays

A non-radioactive cell proliferation assay (MTT or MTS) was used to evaluate the cytotoxicity of TTRV30M preparations. The results obtained for each cell type were similar regardless of the method used, so we calculated the average of all the experiments and respective replicates in which these oligomers were cytotoxic, for each cell type. Preliminary experiments were performed with RPC cells exposed to protein concentrations of 0.5, 1.5 and 3 μM and with an incubation time of 72 hours. A concentration dependent reduction in cell viability was observed (figure 15).

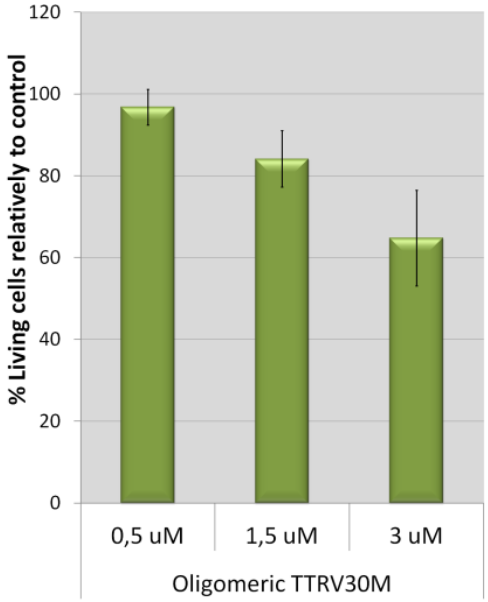


Figure 15 - Non-radioactive Cell Proliferation Assay in the human RPC exposed for 72 hours to concentrations of 0.5, 1.5 and 3 μM of oligomeric TTRV30M. The results are represented as percentage of living cells for each condition relatively to control (cell medium). Values are means of triplicates. Error bar= SD.

In order to reach a compromise between the effect of the protein preparations on the cells and the quantity of protein spent on each experiment, a concentration of 2 μM was chosen for all the subsequent experiments throughout the work.

A modest but statistically significant reduction in cell viability was observed after 24, 48 and 72 hours in the presence of oligomeric TTRV30M when compared to the control (cell media alone), in the five cell types evaluated (figures 16-18 and tables 3-5). Detailed information about the percentages of living cells relatively to controls, and respective standard deviations and student's t-test values are presented in tables 3-5. Comparing cell viability between the oligomeric and tetrameric TTR preparations, the 24 hours incubation time gave more consistent results, showing a statistically significant reduction of cell viability for all cell types in the presence of the oligomeric TTRV30M preparations. Despite being a modest reduction (varying from 7% for RPC to 13% for Hep3B and RPE), oligomeric TTR aggregates reduced cell viability consistently and to a similar amount independently of the cell type, after 24 hours of incubation. Less consistent results were obtained for longer incubation times.

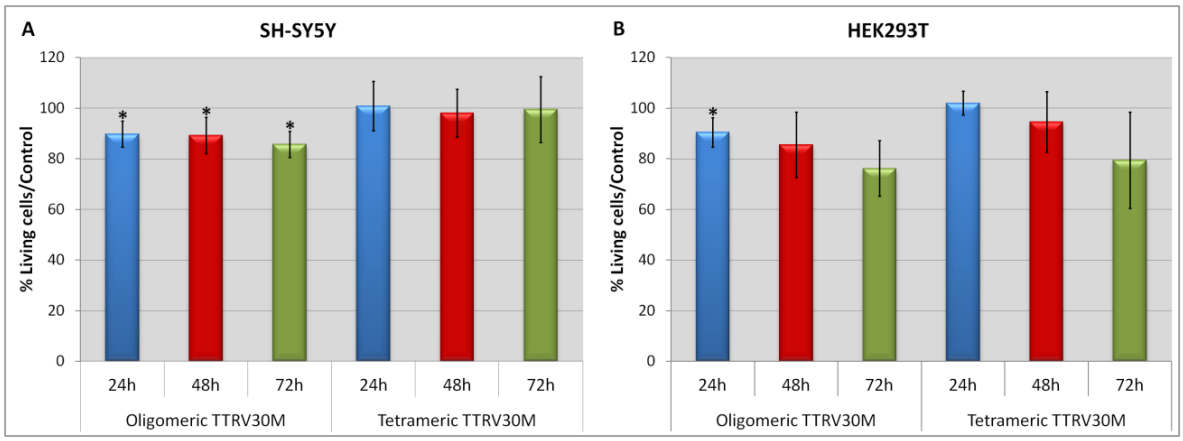


Figure 16 - Non-radioactive Cell Proliferation Assay in the human cell lines SH-SY5Y (A) and HEK293T (B) exposed for 24, 48 or 72 hours to tetrameric and oligomeric TTR V30M preparations. The results are represented as percentage of living cells for each condition relatively to control (cell medium). p value was calculated using student's t-test. *p<0,05 with respect to tetrameric TTR V30M. Values are means of replicates from independent experiments. Error bar= SD.

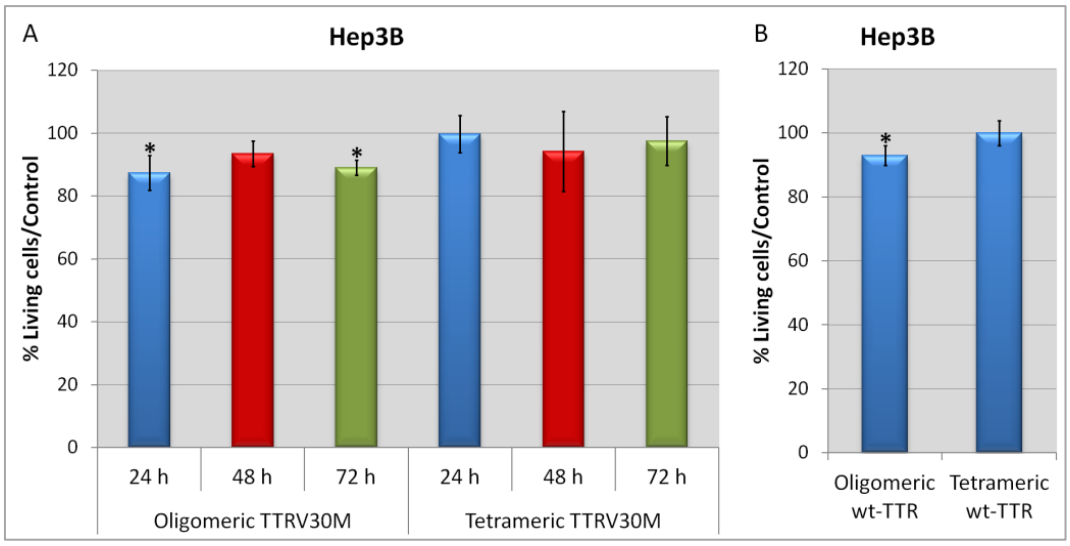


Figure 17 - Non-radioactive Cell Proliferation Assay in the human cell line Hep3B exposed for 24, 48 or 72 hours to tetrameric and oligomeric TTR V30M preparations (A) and for 24 hours to tetrameric and oligomeric wild-type TTR. The results are represented as percentage of living cells for each condition relatively to control (cell medium). p value was calculated using the student's t-test. *p<0,05 with respect to tetrameric TTR V30M. Values are means of replicates from independent experiments. Error bar= SD.

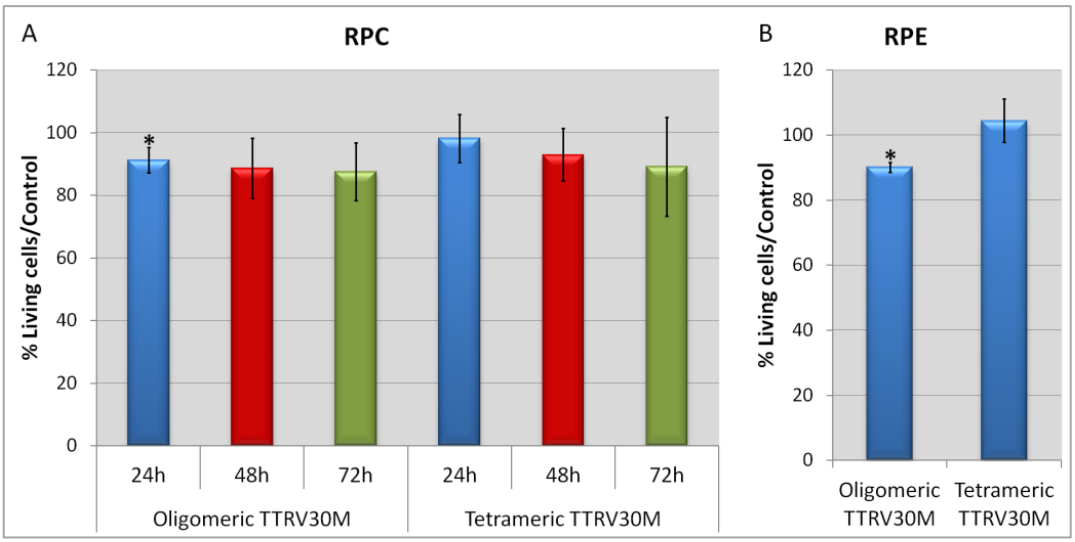


Figure 18 - Non-radioactive Cell Proliferation Assay in human renal progenitor cells (A) and in human retinal pigment epithelial cells (B) exposed to tetrameric and oligomeric TTR V30M preparations, as described on Materials and Methods. The results are represented as percentage of living cells for each condition relatively to control (cell medium). p value was calculated using the student's t-test. *p<0,05 with respect to tetrameric TTR V30M. Values are means of replicates from independent experiments. Error bar= SD.

For 48 and 72 hours the results were not always very reproducible, particularly regarding the tetrameric preparations, which induced toxicity after 48 and 72 hours in HEK293T and RPC cells. In fact, tetrameric TTR may quite possibly be dissociating into monomers during cell culture incubation times longer than 24 hours [278], inducing cell toxicity. The fact that the cytotoxicity induced at longer incubation times occurred particularly on HEK293T and RPC cells may also be related to the varying capacity of different cell types to recover from cellular damage at longer exposure times**,** as described by Cecchi *et al.* using HypF-N toxic aggregates [281].

A preparation of TTRV30M incubated for 72 hours at 37ºC was analysed by DLS and showed that TTRV30M has a tendency to destabilize and form oligomeric species, as shown in figure 14B (section 2 of the Results). Although the oligomers exist in a small percentage (16%) when compared to the tetrameric form, this preparation was also able to induce a reduction of cell viability on Hep3B cells, although to a smaller extent than the preparation that was stirred for 5 minutes (figure 19).

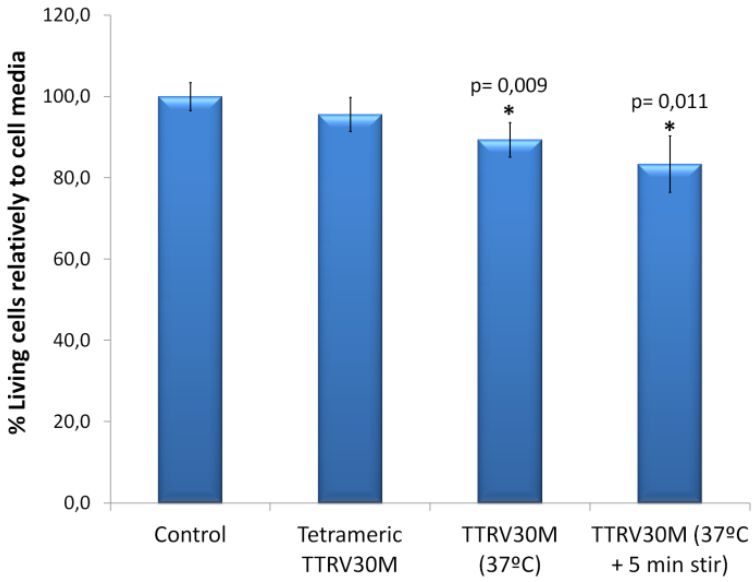


Figure 19 - Non-radioactive Cell Proliferation Assay in Hep3B cells exposed for 24 hours to tetrameric TTRV30M, TTRV30M incubated at 37ºC for 72 h under stagnant conditions and TTRV30M incubated at 37ºC for 72 h under stagnant conditions followed by 5 minutes of stirring. The results are represented as percentage of living cells for each condition relatively to control (cell medium). p value was calculated using student's t-test. *p<0,05 with respect to control. Values are means of triplicates. Error bar= SD.

Oligomers produced from wild-type TTR (wt-TTR) were also evaluated, on Hep3B cells. They induced a reduction of 7.1±3.1% in the Hep3B viability relatively to tetrameric wt-TTR (figure 17B), suggesting that cytotoxicity is induced by oligomeric species regardless of the precursor protein used.

Table 3 – Results of cell viability assays perfomed on human cell lines SH-SY5Y and HEK293T. Cells incubated for 24, 48 or 72 hours in presence of 2 μM tetrameric or oligomeric TTRV30M. Values are means and standard deviations of replicates from independent experiments. p values were calculated using student's t-test.

| | SH-SY5Y | | | | | |
|---|---|---|---|---|---|---|
| | Oligomeric TTRV30M | | | Tetrameric TTRV30M | | |
| Incubation time | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| % Living cells relatively to control | 90±5% | 89±7% | 86±5% | 101±10% | 98±9% | 100±13% |
| p (T-test) relatively to control | $7,2x10^{-7}$ | $5,7x10^{-6}$ | $1,5x10^{-8}$ | 0,70 | 0,40 | 0,87 |
| p (T-test) relatively to tetrameric TTRV30M | $1,6x10^{-4}$ | $1,3x10^{-3}$ | $2,1x10^{-4}$ | - | - | - |
| Number of replicates | 17 | 20 | 14 | 19 | 23 | 20 |
| | HEK293T | | | | | |
| | Oligomeric TTRV30M | | | Tetrameric TTRV30M | | |
| Incubation time | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| % Living cells relatively to control | 90±6% | 86±13% | 76±11% | 102±5% | 95±12% | 79±19% |
| p (T-test) relatively to control | $2,3x10^{-2}$ | $3,9x10^{-3}$ | $2,9x10^{-4}$ | 0,52 | 0,16 | $1,1x10^{-2}$ |
| p (T-test) relatively to tetrameric TTRV30M | $6,5x10^{-3}$ | 0,10 | 0,66 | - | - | - |
| Number of replicates | 5 | 11 | 9 | 7 | 12 | 10 |

Table 4 – Results of cell viability assays perfomed on the human cell line Hep3B. Cells incubated for 24, 48 or 72 hours in presence of 2 μM tetrameric or oligomeric TTRV30M, and for 24 hours in presence of 2 μM tetrameric or oligomeric wild-type TTRV30M (wt-TTR). Values are means and standard deviations of replicates from independent experiments. p values were calculated using student's t-test.

| | Hep3B | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Oligomeric TTRV30M | | | Tetrameric TTRV30M | | | Oligomeric wt-TTR | Tetrameric wt-TTR |
| Incubation time | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 24 h |
| % Living cells relatively to control | 87±6% | 93±4% | 89±2% | 100±6% | 94±13% | 98±8% | 93±3% | 100±4% |
| p (T-test) relatively to control | $6,8x10^{-10}$ | $1,3x10^{-4}$ | $9,5x10^{-4}$ | 0,87 | $8,9x10^{-2}$ | 0,42 | $3,6x10^{-3}$ | 0,50 |
| p (T-test) relatively to tetrameric TTR | $1,1x10^{-8}$ | 0,8 | $5,0x10^{-3}$ | - | - | - | $1,4x10^{-3}$ | - |
| Nº of replicates | 21 | 18 | 7 | 23 | 18 | 11 | 8 | 8 |

Table 5 – Results of cell viability assays perfomed with human renal progenitor cells (RPC) and retinal pigment epithelial cells (RPE). RPC incubated for 24, 48 or 72 hours and RPE incubated for 24 hours in presence of 2 µM tetrameric or oligomeric TTRV30M. Values are means and standard deviations of replicates from independent experiments.

| | RPC | | | | | | RPE | |
|---|---|---|---|---|---|---|---|---|
| | Oligomeric TTRV30M | | | Tetrameric TTRV30M | | | Oligomeric TTRV30M | Tetrameric TTRV30M |
| Incubation time | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 24 h |
| % Living cells relatively to control | 91±4% | 89±10% | 88±9% | 98±8% | 93±8% | 89±16% | 90±2% | 104±7% |
| p (T-test) relatively to control | $8,4x10^{-4}$ | $1,8x10^{-5}$ | $6,9x10^{-4}$ | 0,46 | 0,02 | 0,07 | $8,6x10^{-4}$ | 0,27 |
| p (T-test) relatively to tetrameric TTRV30M | $4,5x10^{-2}$ | 0,15 | 0,77 | - | - | - | $2,0x10^{-2}$ | - |
| Nº of replicates | 9 | 27 | 16 | 8 | 14 | 11 | 4 | 4 |

From these experiments it can be seen that oligomeric TTR aggregates reduced cell viability independently of the cell type after 24 hours of incubation.

Although the results presented here were very consistent, we believe it is important to state that not all the preparations used for the cell culture assays produced cytotoxic aggregates. Some preparations had no effect on the cells, despite being prepared exactly the same way as those that were cytotoxic. We cannot fully explain this observation, but we believe this is due to the particular instability of the protein structure and the stockastic nature of the oligomerization process, that does not always result in the formation of toxic species. These experiments were ignored and the results presented here are the means of replicates from independent experiments in which the oligomeric preparations induced cytotoxicity.

### 3.2. Caspase 3/7 assays

In order to understand if the mechanisms underlying cell toxicity were related to apoptosis, caspases 3/7 assays were performed.

It has been reported that oligomeric aggregates induce apoptosis through activation of caspase 3. So, to understand how cell viability was being compromised in our cell models,

a Caspase-Glo 3/7 assay was used to evaluate the induction of apoptosis by TTRV30M in Hep3B, SH-SY5Y and RPE cells.

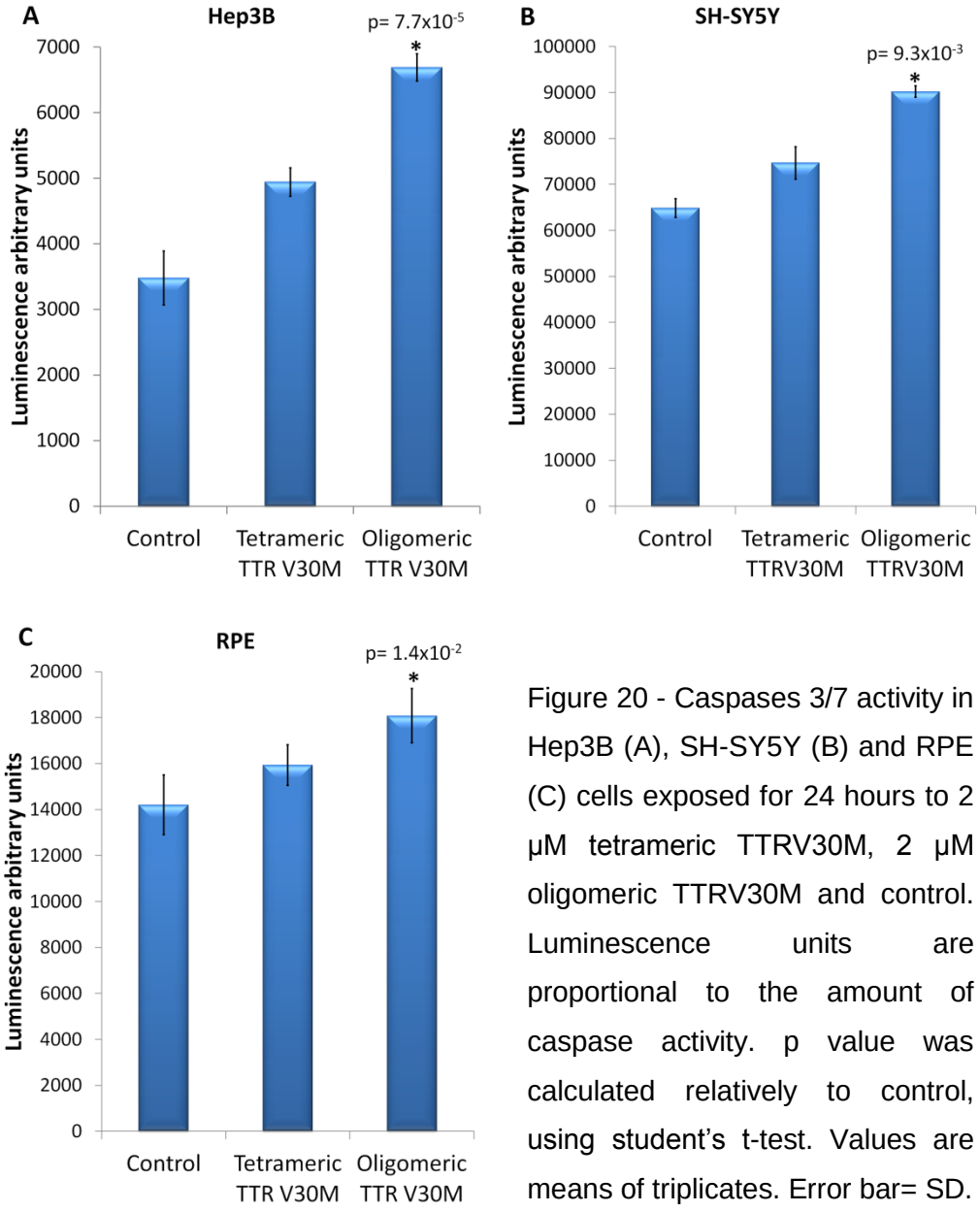


Figure 20 - Caspases 3/7 activity in Hep3B (A), SH-SY5Y (B) and RPE (C) cells exposed for 24 hours to 2 μM tetrameric TTRV30M, 2 μM oligomeric TTRV30M and control. Luminescence units are proportional to the amount of caspase activity. p value was calculated relatively to control, using student's t-test. Values are means of triplicates. Error bar= SD.

Caspase activity was significantly increased in the three cell types exposed to 2 μM oligomeric TTRV30M for 24 hours when compared to cell culture media (figure 20). The most pronounced increase in caspase activity was seen for the Hep3B cells (92.3±6.0%, $p=7.7x10^{-5}$, relatively to cell culture media and 35.4±4.2%, $p=2.4x10^{-5}$, relatively to tetrameric TTRV30M). Increased caspase activity was also observed with exposure to the tetrameric form in the three cell types.

The caspase assay is rather more sensitive than the MTS assay, therefore it is not surprising that some activity is detected after 24 hours of incubation at 37ºC with tetrameric TTRV30M in the caspase assay, but not with MTS. As already mentioned for the viability assays, tetrameric protein solution may begin to form monomers or oligomers that even in small quantities could be sufficient to activate caspases. Our assumption is supported by the fact that DLS of TTRV30M after 72 hours incubation at 37ºC showed that this protein has a tendency form oligomeric species, and was able to induce a reduction of cell viability (figures 14B and 19).

Early oligomeric aggregates have been shown to be more cytotoxic than mature amyloid fibrils [229, 270-272]. Similar reduction levels of cell viability induced by the oligomeric TTRV30M preparations after 24 hours of incubation were observed for the five different cell types evaluated: human hepatoma cell line Hep3B, human neuroblastoma cell line SH-SY5Y, human embrionic kidney HEK293T, human renal progenitor cells (RPC) and primary retinal pigment epithelial cells (RPE). For three of these cell types: Hep3B, SH-SY5Y and RPE, a caspase assay was performed. Together with the reduction of cell viability, a concomitant increase in caspase 3/7 activity was seen in the cells exposed to oligomeric TTRV30M aggregates.

These results agree with previous studies performed using other cell types, mainly neuronal cells (neuroblastoma) [106, 229, 250, 270-272], and primary human umbilical vein endothelial cells (HUVECs) [246], and implicate apoptosis in loss of viability. Interestingly, this cytotoxic effect induced by aggregates seems to be independent of the cell type, contrary to what was reported by Cecchi *et al*. Using aggregates of HypF-N, a prokaryotic peptide domain, that display the same properties of aggregates produced by disease-associated peptides and proteins, they showed that different cell lines have different susceptibilities to damage and apoptosis, and this susceptibility was inversely related to membrane content in cholesterol, to total anti-oxidant capacity of the cell and to the ability of the cell to maintain the balance of intracellular free $Ca^{2+}$ [282]. However, the same group reported later that such differences are more pronounced in longer exposure times due to different recovering capacity from cellular damage [281]. In fact we saw a similar cytotoxic effect between the various cell types in the shortest exposure time to the aggregates. Increasing the incubation time, the results became less reproducible, an increased toxic effect being observed in some cases but a reduction of cytotoxicity in most of them. This may be due to continuing fibrillogenesis, leading to the formation of larger species which may no longer be toxic. According to Cecchi *et al*, the reduction of cytotoxicty at longer incubation times may also be due to recovery from cellular damage.

*In vivo*, it is known that TTR aggregates preferentially deposit in perypheral nerves, causing myelin destruction, and also in the heart, kidney and vitreous rather than in the liver. However, the mechanisms involved in this preference are not yet understood. In view of our citotoxicity results we can speculate that, at least in early disease stages, TTR aggregates may influence cell viability regardless of cell type.

Oligomers generated from wild-type TTR were tested on Hep3B cells and also induced a reduction of cell viability. This result is in agreement with our expectations, as wild-type TTR is also amyloidogenic and responsible for senile systemic amyloidosis (SSA), causing mainly cardiac complications. With these results we can say that both V30M and wild-type TTR oligomeric preparations were cytotoxic, a property that may be common to all prefibrillar amyloid protein aggregates and apparently independent of the target cell type.

### 4. Renal progenitor cell proliferation is inhibited by TTRV30M oligomeric aggregates but maintain their capacity to differentiate into podocytes *in vitro*

A subset of renal progenitor cells (RPC), expressing the surface markers CD133 and CD24, with self-renewal and multidifferentiation potential exist at the urinary pole of Bowman's capsule in the adult human kidney [255]. In Familial Amyloidotic Polyneuropathy, amyloid deposition of mutant TTRV30M leads to renal complications, including nephrotic syndrome and end-stage kidney failure [283].
Here we assessed whether RPC are vulnerable, *in vitro*, to TTRV30M oligomers in order to understand if their regenerative potential could be compromised.

We showed (section 3.1.) that oligomeric TTRV30M aggregates reduce RPC viability by 9±4% after 24 hours. This reduction was significant relatively to both the unexposed and tetrameric TTRV30M exposed controls.
Apoptosis, necrosis and alterations in cell cycle progression were assessed by FACS analysis on cells treated for 48 and 72 hours. TTRV30M oligomers did not induce apoptosis, necrosis or alterations in cell cycle progression to the RPC cells in a significant extent, neither at 48 or 72 h (figure 21).

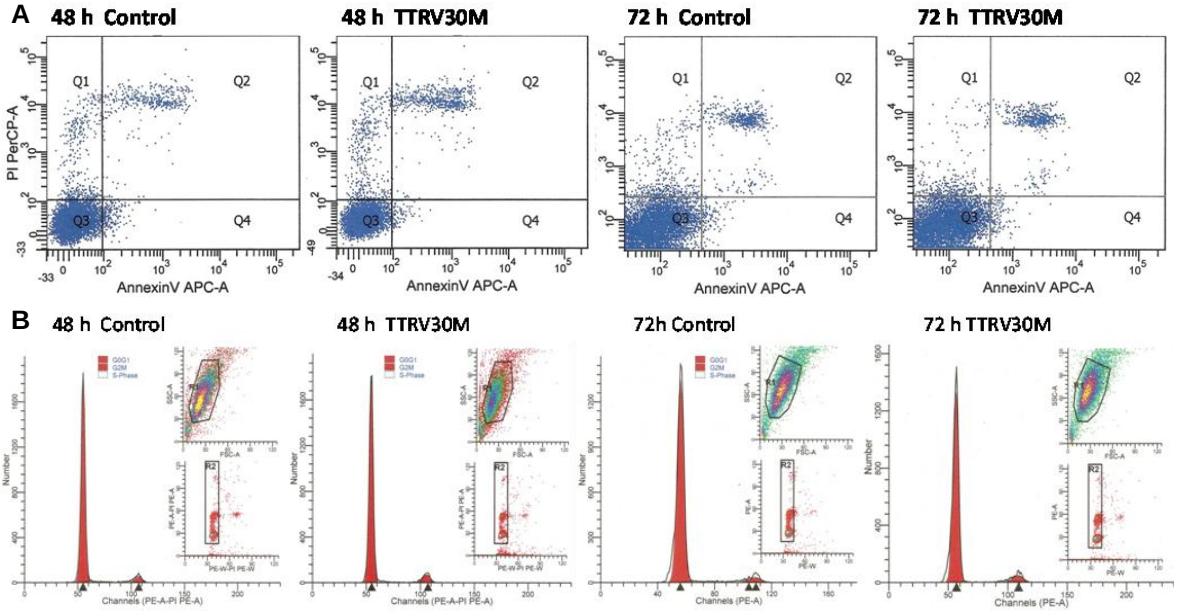


Figure 21 - FACS analysis to evaluate apoptosis and necrosis (A) and alterations in cell cycle (B) in RPC treated with 2 µM oligomeric TTRV30M for 48 and 72 h.

CD133+CD24+ RPC have an inherent capacity to differentiate into podocytes when cultivated in VRAD medium. The influence of oligomeric TTRV30M on the regenerative capacity of these progenitor cells was assessed.

Podocytes express nephrin so the relative expression level of this marker was evaluated by real-time pcr to confirm differentiation. The results varied between experiments, but on average we obtained similar results between the controls and the cells exposed to TTRV30M aggregates, both on differentiation and non-differentiation media (Figure 22).

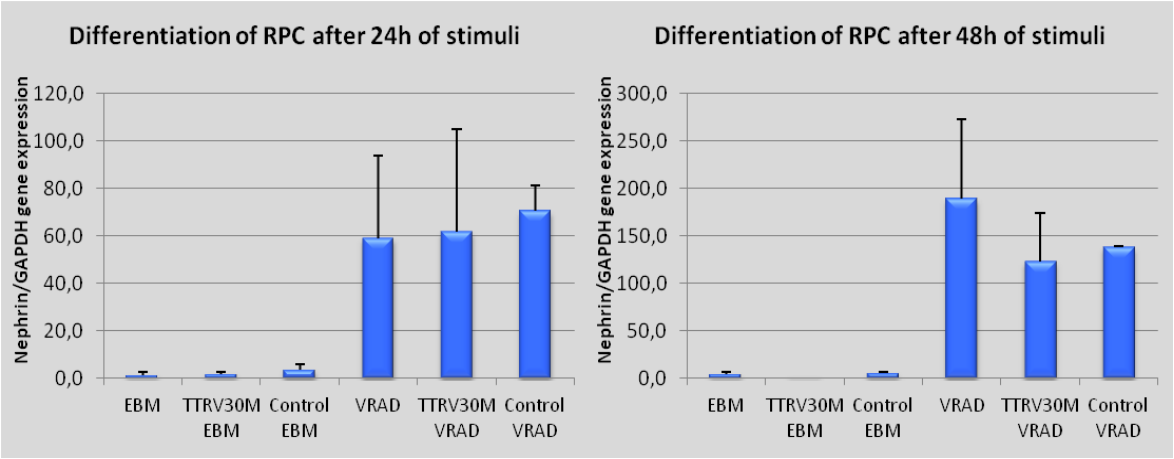


Figure 22 - Differentiation of renal progenitor cells into podocytes. Assessment by quantitative RT-PCR of fold increase mRNA levels for the podocyte marker nephrin and the housekeeping gene GAPDH.

TTRV30M aggregates reduced proliferation of renal progenitor cells. However, in the apoptosis and cell cycle progression assays no significant alterations were observed. Also, in the differentiation experiments we saw no alterations in the expression level of the nephrin gene, a marker for podocytes, relatively to controls, so the inherent capacity of these progenitor cells to differentiate into podocytes was apparently not affected by the oligomers. These results are in agreement with a study of Petrakis *et al*. They saw, with a transgenic mouse model of TTRV30M-related amyloidosis, that TTRV30M deposition has deleterious effects on glomerular basement membrane thickness and podocyte foot process width, but without affecting nephrin and podocin gene expression [284].

A study from Neri *et al*, using murine embryonic stem (ES) cells and haematopoietic progenitor (HP) cells exposed to oligomers of Aβ42 showed that ES, but not HP, displayed some impaired viability but their differentiation was not affected by these oligomers [285], which is in agreement with our results.

From this study we can say that TTRV30M oligomers inhibit RPC proliferation but do not influence their capacity to differentiate into functionally mature podocytes, and thus should not compromise tissue regeneration. These resident progenitor cells can also induce regeneration of tubular structures of different portions of the nephron, which can be critical for preventing irreversible renal failure, and could be useful in cell therapies, particularly in FAP.

We have previously shown that EPO expression occurs in distal tubular cells, podocytes and cells of the cortical collecting ducts, in normoxic FAP patients [138]. Although there is no data regarding the ability of RPC to differentiate into EPO-producing cells, they can differentiate into podocytes and tubular cells, which could be important to maintain EPO production.

## 5. Oligomeric TTR V30M aggregates reduce *erythropoietin* mRNA expression

The principal aim of this study was to evaluate the effects of oligomeric TTRV30M aggregates on EPO gene expression, in a cell culture model of EPO production. Due to the lack of an adequate renal EPO-producing cell line, we used the human hepatoma Hep3B cell line, described in 1987 by Goldberg *et al*. as a constitutive and inducible EPO producer, in an oxygen-dependent manner [273]. In a second phase, retinal pigment epithelial cells (RPE) were also evaluated for endogenous EPO gene expression.

### 5.1. Oligomeric TTR V30M aggregates reduce EPO expression in Hep3B cells

*EPO* mRNA expression in Hep3B cells exposed to 2 µM TTRV30M was evaluated by real-time PCR. Threshold cycle (Ct) values obtained for *EPO* in normoxia were 32.68±0.08 for control media, 32.37±0.28 for tetrameric TTRV30M and 33.30±0.06 for oligomeric TTRV30M. In mimicking hypoxia using DMOG, a cell permeable panhydroxylase inhibitor, Ct values for *EPO* were significantly lower: 28.81±0.10 for control media, 28.58±0.40 for tetrameric TTRV30M and 28.37±0.13 for oligomeric TTRV30M. The housekeeping gene TBP was used as an internal control. Ct values for *TBP* did not differ significantly between samples (25.16±0.19). Relative quantification of *EPO* mRNA levels was calculated taking as reference a standard curve for *EPO* and the results were normalized to *TBP*, using a ΔCT model [286]. *EPO* expression after 24 hours was reduced by 50.3±2.8% (p=0,0092) in normoxic Hep3B cells treated with oligomeric TTRV30M when compared to the tetrameric form of the protein (figure 23A).
With DMOG, *EPO* gene expression levels increased 13 to 22 times. In these simulated hypoxia experiments, cells did not show a significant difference in *EPO* expression levels using either tetrameric TTRV30M or oligomeric TTRV30M aggregates (figure 23B).

Hep3B cells were also exposed to 2 µM tetrameric and oligomeric wild-type TTR for 24 hours in normoxia. *EPO* expression was reduced by 22.0±9.1% (p=0,010) in Hep3B cells treated with oligomeric wild-type TTR when compared to the tetrameric form.

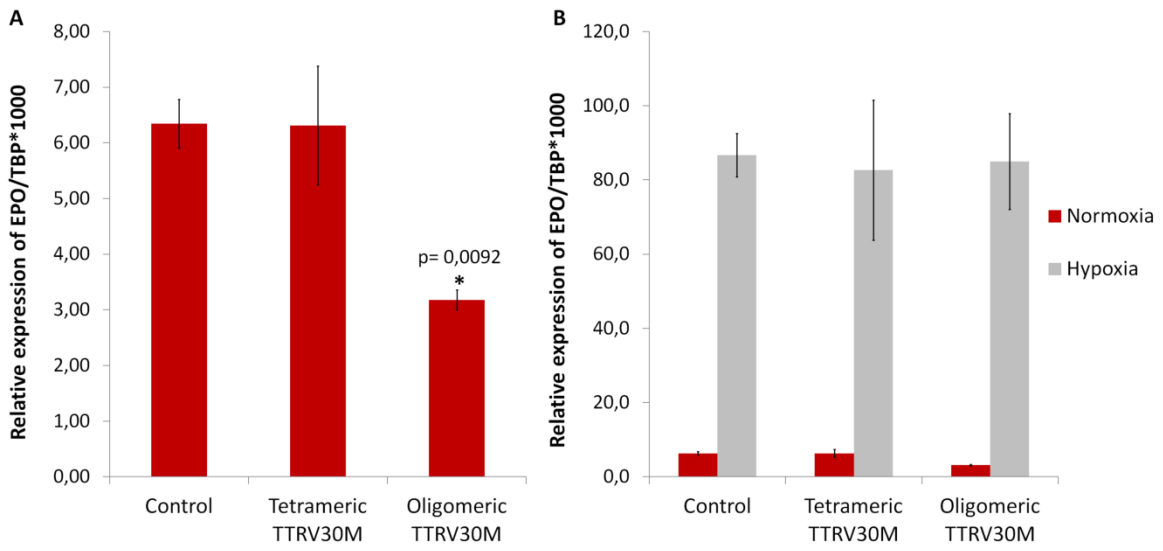


Figure 23 - Relative expression of erythropoietin in Hep3B cells exposed to TTR V30M for 24 hours. (A) Relative expression levels of EPO, normalized for TBP, in normoxia. (B) Relative expression levels of EPO both in normoxia and simulated hypoxia (induced by DMOG). p value was calculated relatively to tetrameric TTR V30M experiments, using student's t-test. *p<0,05. Values are means of duplicates. Error Bar= SD.

The *EPO* gene has a hypoxia responsive element at the *EPO* 3' enhancer site to which hypoxia inducible factor (HIF) can bind [287-289]. In normoxia, the α-chain of HIF is hydroxylated and degraded by the proteasome. In hypoxia the prolyl hydroxylases (PHD) and aspaginyl hydroxylase factor inhibiting HIF (FIH) are inhibited, the α-chain of HIF is not degraded and binds to the β-chain, which is constitutively expressed, and HIF accumulates, inducing gene expression [290-291]. To mimic hypoxia, we used the cell permeable panhydroxylase inhibitor dimethyloxalylglycine (DMOG).

We found that exposure of normoxic Hep3B cells to oligomeric TTR V30M significantly reduces *EPO* mRNA expression. However, Hep3B cells treated with DMOG did not show significant differences in *EPO* mRNA levels when exposed to tetrameric or oligomeric TTR V30M. Under these simulated hypoxia conditions, Hep3B cells up-regulated *EPO* expression 13 to 20 times. It is possible that this large increase in *EPO* expression masks any effect of exposure to TTR aggregates on the gene expression level, or, alternatively, different regulatory mechanisms of EPO production could be involved in normoxia and hypoxia, altering the cellular response to the presence of oligomeric aggregates.
Our previous work using patient kidney biopsies evaluated *EPO* expression in normoxia, and suggested that differences in the regulation of this gene in normoxia and hypoxia are

possible [138]. Recently, Nagai *et al*. explored this subject and demonstrated that, in mice, *EPO* mRNA expression occurs in renal tubular cells in normoxia, as we have shown for the human ATTRV30M amyloidosis kidney, while in hypoxia it occurs largely in peritubular cells, strengthening the case for different regulatory mechanisms for *EPO* expression in normoxia and hypoxia [141].

Anemia in FAP patients sometimes precedes overt clinical disease and persists even after liver transplantation. There is evidence that after liver transplantation, the wild-type TTR can still form amyloid deposits and that there is progression and maybe even acceleration of amyloid deposition [59, 292]. In order to understand the persistence of low EPO production after liver transplantation, we produced oligomeric aggregates from normal TTR. We saw that Hep3B cells exposed to oligomeric wild-type TTR aggregates are also capable of reducing EPO mRNA expression when compared to tetrameric TTR controls. Although these studies were performed *in vitro* with a hepatoma cell line, they support the hypothesis that cytotoxic oligomeric species may be involved in the genesis of anemia in FAP patients. Besides this evidence, we recently showed that EPO concentrations in the aqueous humor of glaucomatous eyes of non-amyloidotic patients are significantly increased relatively to normal non-glaucomatous eyes, probably with a protective role [116]. However, in glaucomatous eyes of FAP ATTRV30M patients the EPO levels did not increase and maintained similar levels to those of control eyes [116]. These results show an inability of these patients to upregulate EPO production also locally in the retina.

### 5.2. Oligomeric TTR V30M aggregates reduce EPO expression in RPE cells

Beyond using Hep3B cells, a tumoral cell line, primary cell cultures of retinal pigment epithelial cells (RPE) were also evaluated for EPO gene expression. RPE cells when are cultivated acquire a hexagonal conformation and are pigmented (figure 24). As the cells divide, they gradually lose their pigment and start differentiating, acquiring a more elongated and fibroblast-like conformation (figures 25 and 26). Preliminary results showed that RPE isolated from the eye of an adult human cadaveric donor express EPO mRNA, but only on early passages (P0 and P1).

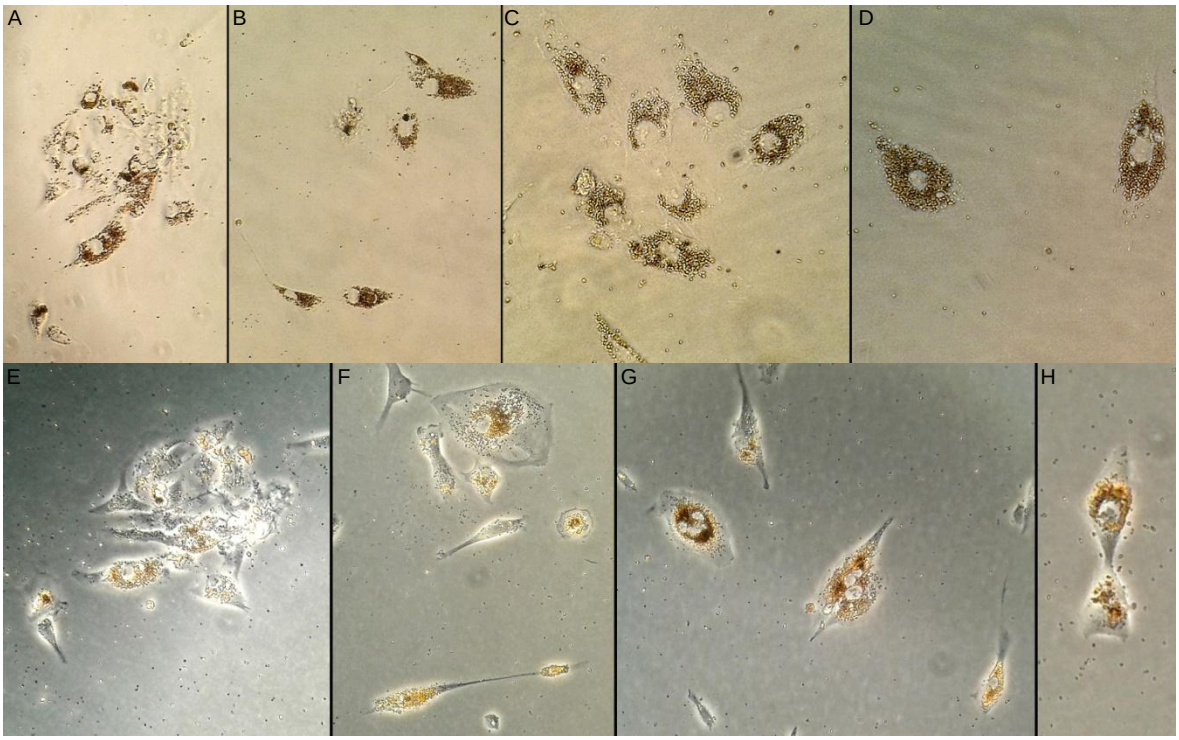


Figure 24 – Day 1 of a culture of retinal pigment epithelial cells (RPE) at passage 1 (P1), cultured on 25 $cm^3$ flasks, visualized under light microscope with original magnifications x100 (A and B) and x200 (C-H). Note the abundance of pigment in the cytoplasm of these cells.

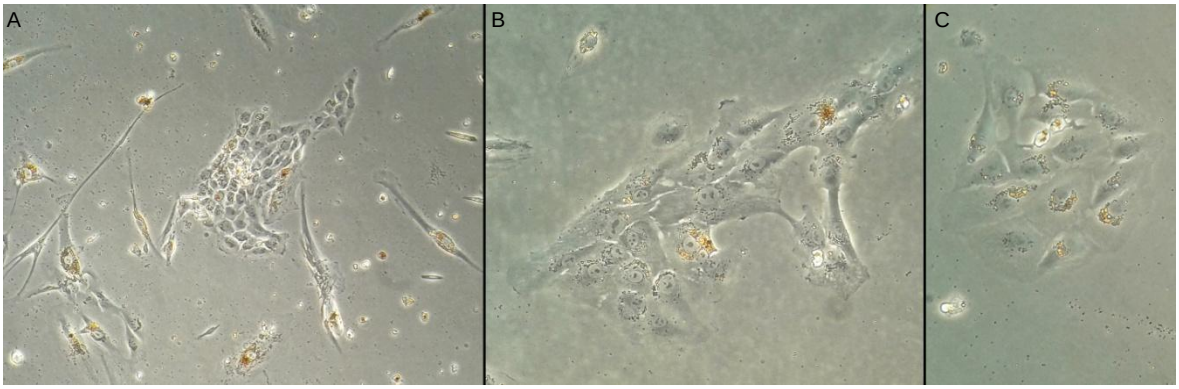


Figure 25 – Day 3 (A) and day 8 (B and C) of a culture of retinal pigment epithelial cells (RPE) at passage 1 (P1), cultured on 25 $cm^3$ flasks, visualized under light microscope with original magnifications x100 (A) and x200 (B-C). As the cells divide they start loosing their pigment.

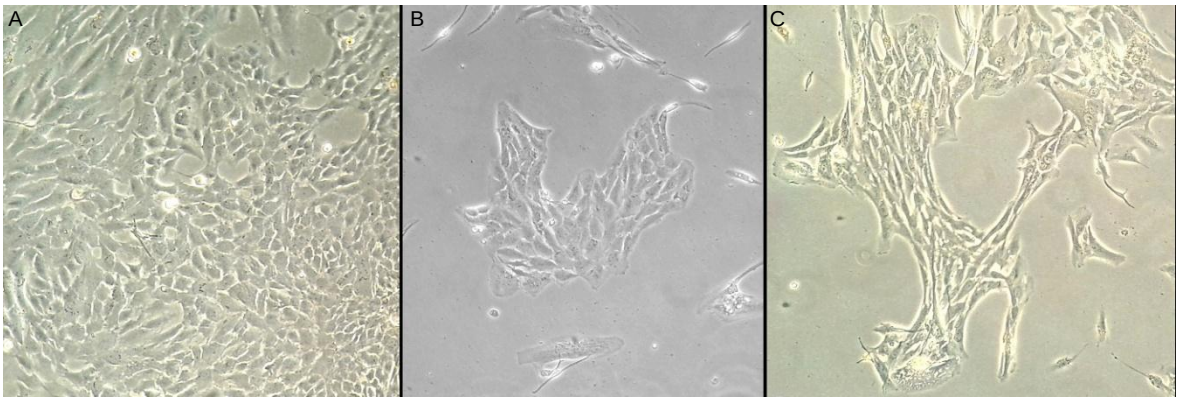


Figure 26 – RPE cells at passage 2 (P2), cultured on 25 $cm^3$ flasks, visualized under light microscope with original magnifications x100. At this passage the cells lost most of their pigment and aquired a more fibroblast-like conformation.

RPE cells on passage 1 were exposed to TTRV30M preparations. As already shown in sections 3.1. and 3.2., oligomeric TTRV30M aggregates reduced RPE cell viability with concomitant increase in caspase 3/7 activity. Real-time PCR showed that, similarly to what happened for Hep3B cells, *EPO* expression after 24 hours was reduced by 48.3±17.1**%** (p= $7.0x10^{-3}$) in normoxic RPE cells treated with oligomeric TTRV30M when compared with tetrameric form experiments (figure 27).

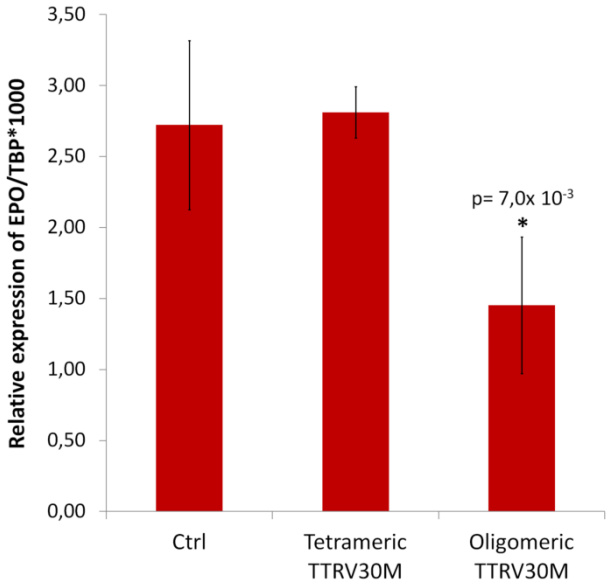


Figure 27 - Relative expression levels of erythropoietin in normoxic RPE cells exposed to TTRV30M for 24 hours. Values were normalized for TBP. p value was calculated relatively to tetrameric TTRV30M, using student's t-test. *p<0,05 with respect to TTRV30M. Values are means of duplicates. Error Bar= SD.

These are unpublished preliminary results. Further experiments are needed to validate these results and to better characterize this primary cell culture model, which responded to DMOG with a significant increase in EPO expression. As RPE cells are producers of both TTR and EPO in the eye, it may be a good model for a more detailed study of the mechanisms behind the inhibition of EPO production in FAP patients.

### 6. Oligomeric TTR V30M aggregates inhibit EPO promoter activity

EPO gene expression is regulated at different levels: transcription, mRNA stabilization and at the translational level by an upstream open reading frame [160-163]. In hypoxia, HIF is the main regulator by binding to a hypoxia responsive element in the EPO 3' enhancer and inducing EPO expression. In normoxia, GATA-2 and NF-kB inhibit EPO expression through proximal promoter binding sites.The transcription factors HIF, NF-kB and GATA-2 are key regulators of EPO gene expression [164-167].

We saw that oligomeric TTR significantly reduces EPO mRNA expression in normoxic Hep3B cells. As in normoxia EPO expression is regulated mainly by NF-KB and GATA-2 transcription factors, we constructed a reporter system containing a fragment of the EPO promoter sequence encompassing their binding sites.

#### 6.1. Transfection of Hep3B cells with Epo-Prom-pGL3

The Epo-Prom-pGL3 reporter construct has recognition sites for the transcription factors GATA-2 and NF-kB in the cloned *EPO* promoter fragment. This reporter system was used to evaluate the influence of TTRV30M on the activity of the EPO promoter.
Transfected Hep3B cells were exposed for 24 hours to different TTRV30M preparations. A significant reduction of the erythropoietin promoter activity (53.1±6.5%, p=0.0043) was observed in the cells exposed to oligomers when compared to cells exposed to the soluble tetrameric form of TTRV30M (Figure 28). No significant effect of tetrameric TTRV30M on the activity of the promoter was observed.

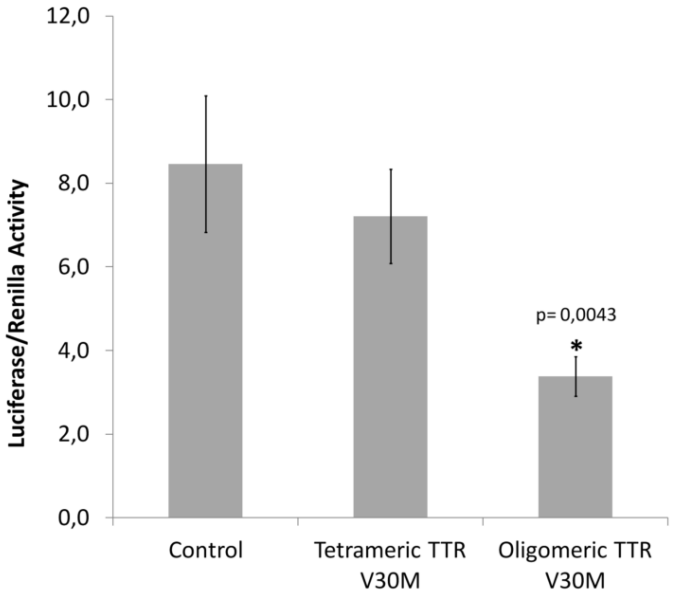


Figure 28 - Hep3B cells transfected with EPO Promoter (Epo-Prom-pGL3) and exposed to TTR V30M for 24 hours. Luminescence levels of Epo-Prom-pGL3 were normalized to Renilla luciferase plasmid pTK-RL. p value was calculated relatively to tetrameric TTR V30M, using the student's t-test. *p<0,05 with respect to TTR V30M. Values are means of duplicates. Error bar = SD

Inhibitory modulation of EPO gene expression by GATA-2 and NF-kB transcription factors, the main known negative regulators at the promoter level, has been demonstrated previously [166-168]. GATA-2 is activated by IL-1 and TNF-α, by exogenous and endogenous $H_2O_2$ and by L-NG-monomethylarginine (L-NMMA), a nitric oxide synthase (NOS) inhibitor that is markedly elevated in uremic patients, with consequent inhibition of EPO gene expression [168, 177-180]. NF-kB upregulation through RAGE, or upregulation of both NF-kB and GATA-2 mediated by oxidative stress and/or inflammatory signaling, are likely mechanisms for the reduction of EPO gene expression induced by oligomeric TTRV30M species. Evidences suggest that Hep3B cells express RAGE [293-294] and interaction of RAGE with transthyretin triggers NF-kB activation [251]. NF-kB may also inhibit EPO expression by inducing a transformation of the renal EPO-producing cells (REP) to myofibroflasts [181]. This phenotypic switch of REP cells to a pathological fibrogenic state is induced by inflammatory injury, and reverses when the inflammatory stimulus ceases.

To explore the role of the EPO promoter in our model, a reporter assay system was constructed with a PCR fragment of the promoter linked to the luciferase gene. We saw a

reduction of EPO promoter activity in transfected Hep3B cells exposed to oligomeric species, parallel to the observed reduction in native EPO mRNA expression. These results suggest that downregulation of EPO expression in normoxic Hep3B cells exposed to oligomeric TTRV30M could be explained, at least in part, by a reduction of EPO promoter activity. As GATA-2 and NF-kB may repress EPO promoter activity, these transcription factors may be involved in the repression of EPO expression and promoter activity induced by the oligomeric TTR aggregates.

### 6.2. Study of NF-kB and GATA-2 protein expression

Immunofluorescence and immunohistochemistry were performed in order to explore the role of NF-kB and GATA-2. Hep3B cells were exposed to TTRV30M preparations for 24 hours and immunofluorescence was performed for both NF-kB and GATA-2. No significant differences were observed between the oligomeric-treated and tetrameric-treated cells.

Immunohistochemistry for NF-KB and GATA-2 was performed on renal biopsies from FAP patients and from renal donors. As for immunofluorescence, no significant differences were observed between the renal FAP biopsies and the donors. Interestingly, GATA-2 antibody cross-reacted with the amyloid deposits of the FAP renal biopsies and gave false-positive results.

The influence of TTR aggregates on the activation of NF-kB and GATA-2 transcription factors still needs clarification. A technique such as chromatin immunoprecipitation (ChIP), followed by real-time pcr, may better elucidate if an interaction between one or both of these transcription factors with the EPO promoter is involved in the inhibition of EPO expression in FAP.

A similar situation to that found in FAP patients, inappropriately low EPO production, also occurs in diabetic nephropathy (DN) [295]. Anemia occurs early in the course of DN due to either impaired production and/or response to EPO, even in the absence of renal impairment [295].
Diabetic nephropathy (DN) is a chronic disorder present in approximately 25%-40% of patients with long-standing diabetes. It is characterized by progressive albuminuria and a decline in renal function [296]. Chronic elevated blood glucose together with glomerular

hypertension leads to renal inflammation, progressive glomerulosclerosis and tubulointerstitial fibrosis resulting in organ failure.

Among the key modulators implicated in the pathogenesis of DN, which adversely affect the response to anemia, are oxidative stress and overproduction of reactive oxygen species (ROS), activation of protein kinase C and mitogen-activated protein kinase (MAPK) signaling pathways, production of advanced glycation end products (AGEs) and activation of the receptor for advanged glycation end products (RAGE) and upregulation of the nuclear factor-κB (NF-κB) [155, 295-297]. In particular, there is excess accumulation and exposure of the kidney to AGEs [298]. AGE may activate downstream targets such as cytokines and growth factors: monocyte chemo attractant protein (MCP-1), transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF) that may be involved in the development and progression of DN. The upregulation of NF-κB may influence EPO production, as this transcription factor suppresses EPO expression [168].

Evidences suggest that, in pre-symptomatic FAP patients, the presence of early non-fibrillar TTR aggregates induces the expression of oxidative stress, apoptosis related molecules and pro-inflammatory cytokines, via interaction with RAGE and activation of NF-kB. A common mechanism that explains the inhibition of EPO production may be involved in both pathologies, FAP and DN, which may be related to the activation of RAGE.

### 6.3. Co-transfection of Hep3B cells with Epo-Prom-pGL3 and pCG-ATF3

Activating transcription factor 3 (AFT3) is a member of the ATF/cAMP responsive element binding protein (ATF/CREB) family. ATF3 is rapidly induced upon exposure of cells to stress signals, such as ischemia, wounding, toxicity, seizures, serum factors, cytokines, genotoxic and cell death-inducing agents [183, 299].

Although ATF3 is not classically described as a regulator of EPO expression, it is involved in the activation of EPO promoter in stromal cells in response to the platelet-derived growth factor (PDGF) signaling system [182]. Besides, overexpression of ATF3 alone led to a substantial increase in EPO promoter activity, although not as potent as PDGF-BB treatment [182].

The role of ATF3 was explored in our model of EPO expression. Hep3B cells were co-transfected with both the Epo-Prom-pGL3 reporter construct and with an ATF3 expression plasmid (pCG-ATF3). This ATF3 plasmid was kindly donated by Professor Tsonwin Hai and is well characterized [300].

Using Hep3B cells transfected with the Epo-Prom-pGL3 reporter construct, it was shown previously that oligomeric TTRV30M aggregates reduced approximately 2-fold the activity of EPO promoter relatively to the tetrameric-treated cells. The overexpression of ATF3 in Hep3B cells co-transfected with the Epo-Prom-pGL3 reporter construct and ATF3 vector led to an increase of the promoter activity in oligomeric-treated cells to a similar value to that observed in tetrameric TTR-treated cells (figure 29).

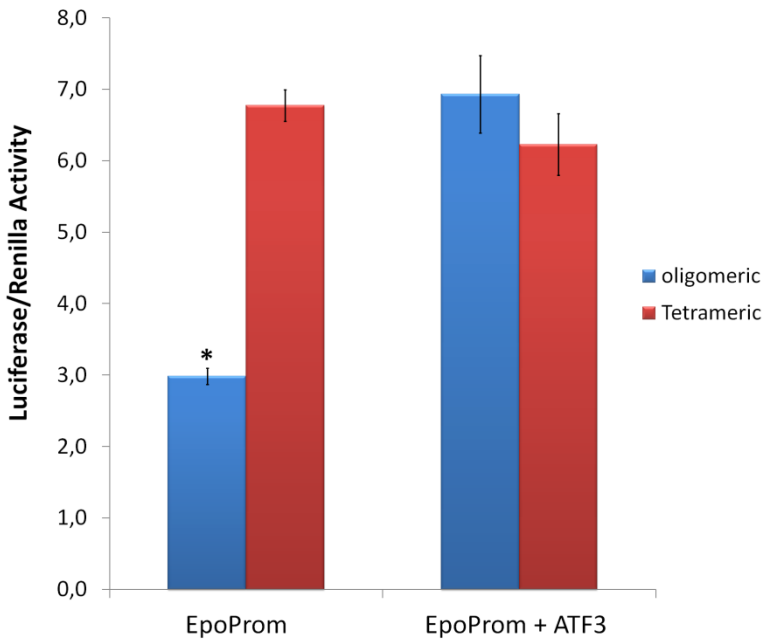


Figure 29 - Hep3B cells co-transfected with EPO Promoter (Epo-Prom-pGL3) and pCG-ATF3, an expression vector containing the ATF3 gene. Cells were exposed to TTR V30M for 24 hours. Luminescence levels of Epo-Prom-pGL3 were normalized to Renilla luciferase plasmid pTK-RL. Error bar = SD

The inhibitory effect induced by TTR oligomers on EPO promoter activity seems to be reversed by the expression of ATF3. These results are preliminary and require further study, but raise the possibly that ATF3 alone, or together with other transcription factors, participates in the regulation of EPO gene expression.

It is known that ATF3 is involved in the regulation of several genes, not necessarily via direct binding to the promoter of the target gene, but forming complexes with other transcription factors or cofactors [301]. It may stabilize the co-factors at the promoter or

sequester them away from the transcription machinery, resulting in transcriptional regulation [182-183, 299-302].
Xue *et al* propose that the formation of a complex between the ATF3, c-Jun and Sp1 explains the increase of EPO promoter activity induced by PDGF-BB [182]. They showed that the EPO promoter region does not contain an ATF3 binding site, suggesting that ATF3 may not act directly on the EPO promoter [182]. Elevated expression of PDGF has been observed in RPE cells after retinal detachments or retinal laser treatment in murine model systems [184], in *in vitro* wounded human RPE cell cultures [185] and in epi-retinal membranes isolated from proliferative vitreoretinopathy and proliferative diabetic retinopathy patients [186].
Besides Sp1 and c-Jun, ATF3 may also interact with NF-kB, p53 and p73. It may also inhibit transcription of pro-inflammatory cytokines, by interacting with NF-κB at the promoter of these target genes [301].

In our TTRV30M exposure Hep3B cell model, it appears that oligomeric TTRV30M aggregates inhibit EPO expression by decreasing EPO promoter activity. However, when the cells over-express ATF3 the inhibitory effect of these aggregates is reverved. This could be due either to increased activity of promoter inhibitors or to a decreased activity of ATF3, preventing it from sequestering these inhibitors away from the EPO promoter. This issue deserves further study.

ATF3 has a basic region-leucine zipper (bZip) DNA binding domain. However, several isoforms derived from alternative splicing have been described (ATF3ΔZip, ATF3ΔZip2a, ATF3ΔZip2b, ATF3ΔZip2c, ATF3ΔZip3, and ATF3b), lacking the leucine zipper region [302]. The role of these alternative splicing forms needs also to be explored.

# CONCLUSIONS AND FUTURE PERSPECTIVES

# Conclusions and Future Perspectives

In this project we carried out *in vitro* studies aiming to explore the role of amyloid deposition in ATTRV30M amyloidosis, especially regarding the role of early non-fibrillar aggregates on cell differentiation and erythropoietin production.

First, the production of recombinant human TTRV30M and their oligomeric aggregates were optimized. Obtaining a pure and LPS-free protein for the cellular assays was crucial to ensure that the effects on the cells were only due to the protein preparations.
The poor reproducibility of preparation of cytotoxic oligomeric solutions made it necessary to test several methods described in the literature, for optimization. Mild acidification (pH 4 to 5.5) of the protein solution, acidification to pH 2.0 followed by NaCl or aging under physiological pH and temperature followed by vigorous stirring were the methods chosen. In the case of acidification to mild pH we obtained insoluble material, probably amorphous aggregates that, despite being positive in ThT assays, were not toxic to cells. The method that induced the formation of most cytotoxic species and with the most reproducible results was the last one tested, aging the protein at physiological pH and 37ºC for 72 hours followed by 5 minutes of stirring. This TTR preparation contained mainly oligomers of approximately 300 nm when analysed by dynamic light scattering, and was the one used for the subsequent cell culture assays.

Different cell culture models were used to evaluate the influence of oligomeric TTR aggregates on cell viability: using immortalized human hepatoblastoma Hep3B, neuroblastoma SH-SY5Y and embryonic kidney cells HEK293T, as well as primary retinal pigment epithelial cells RPE and human renal progenitor cells RPC. The proliferation of the cells exposed for 24 hours to oligomeric TTRV30M aggregates was moderately reduced when compared to the cells exposed to the tetrameric protein, independently of the cell type. Oligomers generated from the wild-type TTR also reduced cell proliferation in comparison to the soluble tetrameric form. Both oligomeric TTR and TTRV30M preparations were cytotoxic, a process that may be common to all prefibrillar amyloid protein aggregates.
Concomitantly to the reduction in cell proliferation, a significant increase in caspases 3/7 activity occurred in cells exposed to oligomeric TTRV30M aggregates, implicating apoptosis in the loss of viability. These results are in agreement with other studies performed mainly in neuronal cells (neuroblastoma) [106, 229, 250, 270-272]. Interestingly, the cytotoxic effect induced by the aggregates seems to be independent of

the cell type, since both immortalized, as well as primary and progenitor cells were shown to be susceptible to damage and apoptosis driven by aggregate exposure.

Renal progenitor cells (RPC) express the stem cell markers CD133+CD24+ and may potentially differentiate into podocytes and tubular cells. We evaluated the influence of the oligomeric aggregates on the viability and differentiation capacity of the RPC, in order to understand if the misfolded protein could compromise a potential therapeutic use of these cells to treat renal damage. Proliferation of RPC *in vitro* was impared by the oligomeric TTRV30M aggregates comparatively to the tetrameric form, but their differentiation capacity into mature podocytes was not significantly affected, so renal tissue regeneration should not be compromised by the presence of early oligomeric species.

ATTRV30M amyloidosis patients develop anemia with low EPO levels. The impaired EPO production is not related to the extent or pattern of congophilic renal deposition or with the presence of circulating mutant TTRV30M. The role of oligomeric aggregate cytotoxicity on EPO production had not been studied yet.

In this work we used the EPO producing cell line Hep3B to evaluate the influence of oligomeric TTRV30M aggregates on EPO gene expression, as well as on pathways linked to the regulatory regions of the EPO gene. Normoxic Hep3B exposed for 24 hours to early oligomeric TTRV30M aggregates showed a 2-fold reduction in EPO mRNA expression. In accordance to this reduction, there was a 2-fold reduction in EPO promoter activity in transfected Hep3B cells. These results support involvement of early oligomeric TTR aggregates in decreased EPO gene expression and could explain the anemia and low EPO levels in FAP patients, seen even in the pre-symptomatic phase of the disease. Interestingly, a preparation of wild-type TTR oligomers also reduced EPO mRNA expression in normoxia, although to a less extent than the TTRV30M oligomers, which may explain the continuing low EPO production after liver transplantation seen in FAP patients.

The inhibition of EPO expression caused by exposure of the cells to cytotoxic oligomeric aggregates seems to be mediated by decreased activity of the EPO promoter. Transcription factors such as NF-kB and GATA-2 bind to the promoter and inhibit transcription of the EPO gene, mainly in inflammatory states. Here, we obtained inconclusive results about the influence of oligomeric aggregates on NF-kB or GATA-2 activation. However, we believe this issue deserves further consideration, as well as other signaling pathways that may be activated by the oligomeric species and that may influence EPO expression.

FAP patients are also unable to up-regulate EPO production in the glaucomatous eye, as a neuroprotective response. Besides the possible involvement of TTR aggregates in EPO expression and anemia, a much wider role for these aggregates in disease progression could be considered. Recent studies point to a potentially major role of EPO in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis and motor neuron diseases [151, 303-311]. It has been already shown in another model of amyloid-related neurodegeneration, in which PC12 cells are exposed to $A\beta_{25-35}$ aggregates, that apoptosis is counteracted by EPO signaling [312-313]. EPO has also been found to increase the resistance of neurons to damage induced by pro-inflammatory agents [314]. It could be postulated that deposition of TTR aggregates in the peripheral nervous system down-regulates local EPO expression, depriving neurons from needed neuroprotective signals, contributing thus for the onset and/or progression of the disease, an hypothesis that we feel deserves further study.

Retinal pigment epithelial cells (RPE) produce both EPO and TTR in the eye. In order to find a more suitable cell culture model to explore the direct effect of the amyloidogenic TTRV30M on EPO production, we isolated RPE cells from an eye of a human cadaveric donor. Preliminary results showed that, at early passages, these cells express EPO in normoxia and respond to hypoxia simulated with DMOG.
Besides the reduction of cell proliferation and increased activity of caspases 3/7, EPO gene expression in normoxic RPE treated for 24 hours with oligomeric TTRV30M aggregates was reduced by approximately 2-fold comparatively to the cells exposed to tetrameric TTRV30M, similarly to what was seen in Hep3B cells. This is an important result as it shows a direct influence of the oligomeric species on a primary cell line that is physiologically responsible for EPO production in the eye.

As future perspectives, and taking into consideration these preliminary results, we believe that RPE cells could become a suitable cell culture model to further explore the mechanisms responsible for EPO inhibition in ATTRV30M amyloidosis, ideally, using RPE cells from FAP patients.

The blockage of EPO production observed both in the kidney as in the eye of FAP patients also happens in Diabetic Nephropathy (DN). Oxidative stress, overproduction of reactive oxygen species (ROS), production of AGEs, activation of the receptor for advanged glycation end products (RAGE), upregulation of the NF-κB and pro-inflammatory cytokines seem to be common factors between FAP and DN. ROS may upregulate GATA-2. In this work we used immunofluorescence to study the activation of

NF-kB and GATA-2 by exposure to oligomeric aggregates and we could not see significant differences. However, it would be important to use more sensitive techniques, such as Chromatin immunoprecipitation or electrophoretic mobility shift assays (EMSA), which can show protein-DNA interaction, to confirm if the inhibition of EPO expression by cytotoxic aggregates is indeed related to activation of these two transcription factors. ATF3 is other transcription factor that may be involved in EPO gene regulation. In our cell model, the over-expression of ATF3 reversed the inhibitory effect of the oligomeric TTR aggregates on the EPO promoter activity. This is a preliminary observation that deserves further study. It would also be interesting to use DN patients for comparison.

Tafamidis is a drug already approved for the treatment of eligible FAP patients. We would like to know if Tafamidis, a TTR tetramer stabilizer, is able to prevent blockage of EPO production.

In summary, although we have shown here that exposure to cytotoxic oligomeric TTR aggregates inhibits EPO expression *in vitro*, much remains to be discovered about the mechanisms underlying this inhibition and possible therapeutic targets.

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# Annex

**Papers published by the author of this thesis during the PhD, that have not been used on the results section, but are in the context of theme developed here**

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# Aqueous humor erythropoietin levels in open-angle glaucoma patients with and without TTR V30M familial amyloid polyneuropathy

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**Purpose:** Glaucoma is the leading cause of irreversible blindness in familial amyloidotic polyneuropathy (FAP) patients. Erythropoietin (EPO) is a cytokine that has been shown to play a role in neuroprotection and is endogenously produced in the eye. EPO levels in the aqueous humor are increased in eyes with glaucoma. In this study, we evaluated the EPO concentration in the aqueous humor of FAP and non-FAP patients, with and without glaucoma.
**Methods:** Undiluted aqueous humor samples were obtained from 42 eyes that underwent glaucoma surgery, phacoemulsification, or vitrectomy. EPO concentration in the aqueous humor and blood were measured using the Immulite 2000 Xpi using an automatic analyzer (Siemens Healthcare Diagnostics).
**Results:** The mean EPO concentration in the aqueous humor of non-FAP glaucoma eyes group 2 (75.73±13.25 mU/ml) was significantly higher than non-FAP cataract eyes (17.22±5.33 mU/ml; $p<0.001$), FAP glaucoma eyes (18.82±10.16 mU/ml; $p<0.001$), and FAP nonglaucoma eyes (20.62±6.22 mU/ml; $p<0.001$). There was no statistically significant difference between FAP nonglaucoma eyes versus non-FAP cataract eyes ($p = 0.23$) and FAP glaucoma eyes versus FAP nonglaucoma eyes ($p = 0.29$). In the glaucoma groups, there was no correlation between the aqueous humor EPO concentration and the ocular pressure ($p = 0.95$) and mean deviation ($p = 0.41$). There was no correlation between the EPO serum concentration and EPO aqueous humor concentration in our patients ($p = 0.77$).
**Conclusions:** Unlike other glaucomatous patients, FAP patients with glaucoma do not show increased and potentially neuroprotective endocular EPO production in the aqueous humor and may need more aggressive glaucoma management.

Glaucoma is a progressive optic nerve neuropathy and the major cause of preventable and irreversible blindness worldwide. It is characterized by visual field defects and nerve head cupping due to the loss of retinal ganglion cells [1]. Despite its multifactorial genesis [2-4], the major risk factor for glaucoma progression is the elevated intraocular pressure (IOP) [5,6], which compresses the retinal ganglion cells at the optic nerve head [7]. The only treatment that slows glaucoma progression involves lowering the IOP [8].

Familial amyloid polyneuropathy (FAP) is caused by the extracellular deposition of amyloid fibrils of mutant transthyretin (TTR) V30M in various tissues and organs [9-11]. TTR V30M mutation is the most common form of transthyretin amyloidosis (ATTR) variant in Portugal as well as in the world [12]. The main clinical expression of FAP disease is a sensorimotor and autonomic neuropathy, but other manifestations, such as nephropathy and hematologic and ocular abnormalities can occur. Among the reported ocular FAP complications [13-15], glaucoma is the major cause of irreversible vision loss and is often difficult to control [16].

Erythropoietin (EPO) was identified as a hematopoietic cytokine that promotes proerythroblast survival and maturation [17]. Recently, EPO was recognized as a member of the cytokine type 1 superfamily with multiple functions outside the bone marrow [18]. It provides direct protection against hypoxia by its anti-apoptotic, anti-oxidative, and anti-inflammatory properties and for its angiogenic capacity that allows the oxygen supply to ischemic tissues. Several studies have found that EPO protects photoreceptor cells, retinal ganglion cells, and retinal pigment epithelial cells from apoptosis [19-26]. Hernandez et al. [27] suggested that EPO is produced locally in the retina. Muller cells and retinal pigment epithelium were identified by Fu et al. [28] and Garcia-Ramírez et al. [29], respectively, as the cells responsible for EPO production in the eye.

Previous studies have shown a significantly increased EPO concentration in the aqueous humor of eyes with glaucoma [30-32]; this is probably a defence mechanism against

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glaucomatous damage [33] caused by hypoxia, ischemia, oxidative stress, and reduced pro-inflammatory cytokine production [34-39]. Although hypoxia/ischemia is the major stimulus for endocular and systemic EPO production [21,40-43], other incompletely understood factors may be involved [27,29].

FAP patients and even presymptomatic carriers have an inappropriately low EPO production [44]. In vitro studies suggest that the dissociated mutant TTR that polymerizes into misfolding amyloidogenic intermediates, protofilaments, and nonfibrillar aggregates of TTR rather than mature amyloid fibrils may induce cellular toxicity [45,46]. We propose that these amyloid precursors may be toxic to EPO-producing cells. This study was performed to evaluate the ocular EPO response in FAP patients with glaucoma.

## METHODS

It was recruited 42 eyes of 42 patients (18 females) with a mean age of 56.8±7.4 years. A prospective, controlled, nonrandomized, nonblind comparative study was conducted from January 2008 to December 2011 at the Ophthalmic and Clinical Chemistry Departments from Centro Hospitalar do Porto, Porto. Written informed consent was obtained from all patients. This study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Ethics Committee of the Centro Hospitalar do Porto.

Presurgical assessment included Snellen best-corrected visual acuity (Snellen chart, Takagi chart projector CP-30, calibrated for approximately 6 m), slit-lamp biomicroscopy, intraocular pressure (IOP) measurement by Goldman applanation tonometry (same person with AT-900 tonometer; Haag-Streit, Koniz, Switzerland), fundoscopy (90 D noncontact slit-lamp lens; Volk Optical, Mentor, OH), Humphrey perimetry (Humphrey Field Analyzer; Humphrey Instruments, San Leandro, CA), and the cup/disc ratio. All examinations were performed within 2 weeks before the surgical procedure.

Exclusion criteria for all groups were: previous laser and/or intraocular surgery; history of systemic (e.g., diabetes mellitus, kidney disease, cardiovascular disorders, anemia, immune disease, except FAP in groups 1 and 3) or any ocular disorders (e.g., age-related macular degeneration); history of medications that could influence the level of EPO (e.g., iron preparations, chemotherapeutic agents, granulocyte colony-stimulating factor, or systemic therapy with EPO), and patients with any type of glaucoma except open-angle glaucoma, such as angle-closure, pigmented, exfoliation, normotensive, and neovascular glaucomas, or ocular hypertension.

To clarify the relationship between aqueous EPO production and circulating blood EPO levels, we compared the aqueous and serum concentrations of EPO. Aqueous humor samples were obtained from each eye before the beginning of surgery (trabeculectomy, phacoemulsification, or vitrectomy). The standard procedure involved collecting undiluted aqueous humor samples (50–150 µl) through a paracentesis, using a 30-gauge needle on a tuberculin syringe under an operating microscope. Samples were obtained carefully to avoid touching intraocular tissues or blood contamination. All samples were carefully protected from light and were sent immediately to the laboratory for EPO measurement. At the same time, 9 ml of venous blood samples were collected in EDTA tubes from an antecubital vein immediately before sugery. The blood was immediately centrifuged and the blood serum put on the automatic analyzer.

Serum samples were obtained from the centrifugation of the blood sample. The samples of aqueous humor and serum had the same processing routine analysis. Serum and aqueous humor EPO concentrations were measured by a chemiluminescent method in an automatic Xpi Immulite 2000 analyzer (Siemens Healthcare Diagnostics, Siemens AG, Munich, Germany).

*Statistical analysis:* Statistical analysis was performed using nonparametric tests. The Kruskal–Wallis test was used to compare the groups in relation to age, and the chi-square test was used in relation to gender. The Mann–Whitney *U* test was used to compare the nonglaucoma, glaucoma, and FAP groups in relation to aqueous humor EPO and serum EPO levels. The relation between EPO and serum was evaluated by Spearman correlation. Values of $p<0.05$ were considered statistically significant. Data analysis was performed using IBM SPSS Statistics software version 20.

## RESULTS

A total of 21 glaucomatous eyes from 21 patients and 21 control eyes (21 patients) were enrolled in the study. The demographic characteristics of the patients are summarized in Table 1. Of the glaucoma eyes, ten were from FAP patients (group 1, mean age 55.4±10.0 years mean and standard deviation; five females) and 11 were from non-FAP patients (group 2, mean age 55.8±7.0 years mean and standard deviation; four females). Of the 21 control eyes, nine were from FAP patients with an indication for vitrectomy due to amyloid deposition (group 3, 55.9±8.5 years mean and standard deviation; four females) and 12 were from non-FAP patients awaiting phacoemulsification and intraocular lens implantation (group 4, 58.8±4.9 years mean and standard deviation; five females). Groups 1 and 2 presented indications for trabeculectomy,

Table 1. Demographic of the groups.

| Age/Sex | Group 1 FAP glaucoma | Group 2 Non-FAP glaucoma | Group 3 FAP non-glaucoma | Group 4 Non-FAP non-glaucoma |
|---|---|---|---|---|
| Age (year, mean±SD) | 55.4±10.0 | 55.8±7.0 | 55.9±8.5 | 58.8±4.9 |
| Female/Male | 5 / 5 | 4 / 7 | 4 / 5 | 5 / 7 |

Table 2. EPO in aqueous humor and serum of the groups.

| Aqueous humor/ serum EPO level | Group 1 FAP glaucoma | Group 2 Non-FAP glaucoma | Group 3 FAP non-glaucoma | Group 4 Non-FAP non-glaucoma |
|---|---|---|---|---|
| Aqueous humor EPO level (mU/ml) | 18.82±10.16 | 75.73±13.25 | 20.62±6.22 | 17.22±5.33 |
| Serum EPO level (mU/ml) | 13.44±4.82 | 9.99±2.84 | 15.04±5.87 | 8.73±4.12 |

Aqueous humor EPO (Mann–Whitney U test): Group 3 versus Group 4 p=0.23 ; Group 1 versus Group 2 p<0.001; Group 1 versus Group 3 p=0.29; Group 2 versus Group 4 p<0.001

had uncontrolled IOP (defined as IOP higher than the target pressure with maximally topical antiglaucoma medications: prostaglandin + beta blocker + anhydrase carbonic inhibitor + alpha-2 agonist), abnormal visual field test results, and abnormal cup/disc ratio.

The ages and gender distribution of the patients were similar between groups (Kruskal–Wallis test, p = 0.56; chi-square test, p = 0.94). All FAP patients had received an orthotopic liver transplant.

As summarized in Table 2, the mean EPO concentration in the aqueous humor of nonglaucomatous eyes (group 3 versus group 4) was not significantly different between FAP and non-FAP patients (20.62±6.22 mU/ml in group 3 and 17.22±5.33 mU/ml in group 4, p = 0.23) and corresponds presumably to the basal ocular production of EPO. In the presence of glaucoma, EPO concentrations in the aqueous humor showed a significant increase in the non-FAP group (group 2, 75.73±13.25 mU/ml; group 1, 18.82±10.16 mU/ml; p<0.001), and when we compared the non-FAP glaucoma group (group 2) with the nonglaucoma groups (FAP group 3 and non-FAP group 4), a similar finding was observed (p<0.001) (Table 2). In the FAP groups (group 1 and group 3), we observed no significant difference between the mean EPO values of patients with or without uncontrolled glaucoma (p = 0.29). As listed in Table 3, FAP patients with glaucoma (group 1) and non-FAP patients with glaucoma (group 2) were comparable in terms of the IOP (p = 0.39) and mean deviation (p = 0.75). The correlation between the IOP and the aqueous humor EPO was not significant in group 1 (mean IOP 26.20±1.93 mmHg; $r_s$ = 0.02, p = 0.95) and group 2 (mean IOP 26.82±1.72 mmHg; $r_s$ = 0.27, p = 0.41). There was also no significant correlation between the mean deviation and the aqueous humor EPO in group 1 ($r_s$ = –0.48, p = 0.16) or group 2 ($r_s$ = –0.07, p = 0.83).

Serum EPO levels among patient groups were not significantly different when multiple testing was taken into account (Bonferroni correction). No statistically significant correlation between the values of EPO in the serum and in the aqueous humor was observed in any patient (Spearman correlation coefficient r = 0.047, p = 0.77).

Table 3. Intraocular pressure and mean deviation in glaucoma groups.

| IOP/mean deviation | Group 1 FAP Glaucoma | Group 2 Non FAP Glaucoma | P value Mann–Whitney test |
|---|---|---|---|
| N | 10 | 11 | |
| IOP, mmHg, mean±SD | 26.20±1.93 | 26.82±1.72 | 0.39 |
| Mean deviation, dB, mean±SD | −8.92±3.30 | −8.26±3.63 | 0.75 |

## DISCUSSION

Glaucoma is a manifestation of a heterogeneous group of diseases with a very complex and multifactorial pathophysiology [8]. Although hypotensive therapy is today the only possible therapeutic intervention, neuroprotective treatment strategies are emerging as a result of the advances in the comprehension of the pathophysiological mechanisms of glaucoma. In the future, neuroprotective agents will probably be part of the therapeutic arsenal available for the treatment of glaucoma. EPO has been shown to have a protective effect on ganglion cells against acute ischemia injury [28,47] and has been proposed as a potential neuroprotective treatment.

In this study we confirmed that the aqueous humor EPO level is higher in glaucomatous eyes than in nonglaucomatous eyes with cataracts, as previously reported [30-32,48,49]. This increase in aqueous humor EPO levels could be a result of local production and/or active transport through the blood–ocular barrier. This observation lends support to the hypothesis that EPO acts as an endogenous neuroprotector of retinal ganglion cells [19].

In spite of the inappropriately low renal EPO production reported in FAP ATTR V30M [44], its basal level in the aqueous humor of FAP patients was not significantly altered. However, FAP patients seemed to be unable to increase endocular EPO production in the presence of glaucoma. In previous studies, we showed an inappropriate secretion of renal EPO in FAP and an inability to increase EPO production in response to decreased serum hemoglobin levels, leading to a high incidence of anemia in these patients. The lack of response to glaucoma in FAP patients could be the ocular counterpart of the stunted renal EPO production in FAP in response to anemia.

It has been suggested that inhibition of EPO production could be caused by the toxicity of prefibrillar aggregates of TTR V30M [44,50,51]. These oligomers induce the expression of oxidative stress, pro-inflammatory cytokines, and apoptosis-related molecules [52,53] through the binding of TTR aggregates to the receptor for advanced glycation end products, activation of extracellular signal-regulated kinase cascades, and nuclear transcription factor kB [52-56], suppressing the EPO production. All our FAP patients had previously received an orthotopic liver transplant to eliminate their main source of mutant TTR, their own liver [57]. After liver transplantation, mutant TTR is removed from systemic circulation; however, its local production in the eye remains presumably unaffected. Therefore, the ocular pathology related to FAP, which includes glaucoma, continues to progress after liver transplantation; presumably there is also continuing deposition of cytotoxic prefibrillar TTR aggregates.

Garcia-Ramirez found that other factors besides hypoxia-inducible factors (HIF)-mediated hypoxia might be important in the upregulation of EPO. Hypoxia, ischemia, elevated reactive oxygen species, or increases in glutamate and nitric oxide caused by glaucomatous damage are probably the cause of elevated aqueous humor EPO concentration in chronic glaucoma [30]. The pro-inflammatory cytokines interleukin (IL)-1, IL-6, interferon-γ, and tumor necrosis factor (TNF)-α inhibit EPO production [58,59], but despite being increased in the aqueous humor of glaucoma eyes, as is especially the case for TNF-α [60], these cytokines do not prevent an increase in EPO levels.

Increased levels of TTR in the aqueous humor of glaucoma patients have been documented [61-63]. If glaucoma leads to an increase expression of TTR in the aqueous humor, an increased concentration of the unstable TTR V30M in FAP patients' eyes could contribute to the increased development of a mechanical barrier to the outflow of the aqueous humor [64], resulting in worsening the glaucoma. The association of open-angle glaucoma with autonomic nervous system dysfunction suggests that this could also play a role in the pathogenesis of the disease [65]. Patients with systemic sympathetic and parasympathetic neuropathies have a higher incidence of open-angle and normal-pressure glaucoma [66-69]. Because FAP patients have an early onset neuropathy with markedly autonomic involvement, it is likely that autonomic dysfunction plays a role in glaucoma pathophysiology. Other possible contributing factors are the hemodynamic instability often presented in FAP patients due to vascular deregulation and abnormal blood pressure that may compound the harmful effects of glaucoma, particularly during sleep [65].

In the groups with glaucoma, there was no correlation between the aqueous humor EPO concentration and the values of IOP and mean deviation. It seems that the concentration of EPO in the aqueous humor is not related to the IOP in eyes with glaucoma or previous eye injury caused by glaucoma.

In this study, patients with pseudoexfoliative and uveitic glaucomas were excluded because some studies pointed to blood–aqueous humor barrier breakdown in these situations [70,71]. EPO can cross the blood–brain barrier and blood–retina barrier [41]. We did not found a significant correlation between aqueous humor and serum EPO concentrations as other authors have found [30,31]. The elevation of the aqueous humor EPO level in glaucoma was not associated with a parallel increase in blood EPO levels, corroborating the role

of local EPO production as already proposed by Fu [28] and Garcia-Ramirez [29].

In conclusion, our study confirmed that the level of EPO is increased in aqueous humor of open-angle glaucomatous eyes, as found by other authors. This increase was not observed in FAP patients. With the increased survival of transplanted FAP patients, glaucoma prevalence is expected to increase dramatically with increased suvival of the transplanted patients. We showed lower endogenous neuroprotection in glaucomatous eyes of FAP patients, emphasizing the need for more aggressive glaucoma management to maintain vision through life.

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# Use of MALDI-TOF Mass Spectrometry to Assay the Transthyretin V30M Mutation in Serum From a Liver Transplant Donor: A Case Report

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Familial amyloidotic polyneuropathy (FAP), Portuguese type, or ATTR V30M is an autosomal dominant inherited disorder caused by a mutation in the transthyretin gene, with a valine/methionine substitution at position 30 (TTRV30M).[1] ATTR V30M is characterized by a progressive sensory/autonomic polyneuropathy and multiple organ dysfunction.[2]

Liver transplantation is the main therapeutic option,[3] as it virtually eliminates the production of circulating TTR V30M, which occurs predominantly in the liver.

## CASE REPORT

In 2004, a then 35-year-old female FAP patient underwent orthotopic liver transplantation from a cadaveric donor to treat her condition. Progression of FAP after liver transplantation has been documented but usually stabilizes after some time.[4] Continuing deterioration in this patient raised the suspicion, confirmed in 2012, that the liver donor was also a TTR V30M carrier. Retransplantation was proposed and carried out, but these unfortunate circumstances made it necessary to assure the anxious patient, and the transplantation center for that matter, that this second time the transplanted liver was FAP free.

Serum from the second cadaveric transplant donor was sent to us, a few days after the procedure, and absence of TTRV30M was established by immunoprecipitation of TTR followed by mass spectrometry analysis.[5] The spectra that comprise the molecular mass of TTR monomers are represented in Figure 1. In the positive control sample (Figure 1C), we identified 3 peaks that correspond, respectively, to the free form of wild type TTR (13.708 ± 10 Da), the cysteine (Cys) conjugated form of the wild type TTR (13.824 ± 10 Da), and the cysteine conjugated form of the TTR V30M variant (13.860 ± 10 Da), with a difference of approximately 33 Da from the wild type TTR.[5] The liver transplant donor sample (Figure 1A) showed the 2 peaks attributed to wild type TTR only (13.694 ± 10 and 13.816 ± 10 Da) as seen in the negative control (Figure 1B).

## DISCUSSION

Domino FAP liver transplantation into non-FAP patients is common in some centers. In these cases, TTRV30M production has been found to be followed by FAP symptoms as soon as 7 years after transplantation.[6] When this happens, retransplantation is usually proposed. In areas with a high prevalence of FAP, like the north of Portugal (1:550),[7] there is a non-negligible risk of a FAP patient receiving a liver from an untested FAP donor. Portuguese law assumes that every citizen who does not declare otherwise is an organ donor. Unfortunately, reliable individual clinical information is usually not available. Familial amyloidotic polyneuropathy is a late-onset disease, and predictive testing, even if done, is not to be found in clinical records, by force of law. So, at the time of transplantation, up to 12 hours after liver collection, there is usually no other information on personal or family medical history of the donor besides the standard information delivered by the histocompatibility center regarding infectious agents and malign neoplasias.

The risk of transplanting an FAP patient with a TTR V30M positive liver is admittedly low. However, in a country that has performed more than a thousand of these transplant procedures, such an event was to be expected, sooner or later. DNA and serum-based assays for carrier status that could be performed in a timely manner, while not standard, can be easily implemented. It should be noted, though, that the legal and ethical dimensions of performing what would possibly be a predictive genetic test in a deceased donor is unchartered territory, and would have to be addressed. Namely, the question of whether it would be justified to communicate, in the absence of consent, the genetic status of the deceased donor to family members would have to be weighted in terms of

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*Pedro Castro Lacerda and Luciana Moreira wish it to be known that, in their opinion, the first two authors should be regarded as joint first authors'.*

*P.C.L., L.M., and R.V. participated in the research design, carried out the experiments and data analysis, and contributed to writing of the paper. P.P.C. participated in research design, in the data analysis, and in writing of the paper.*

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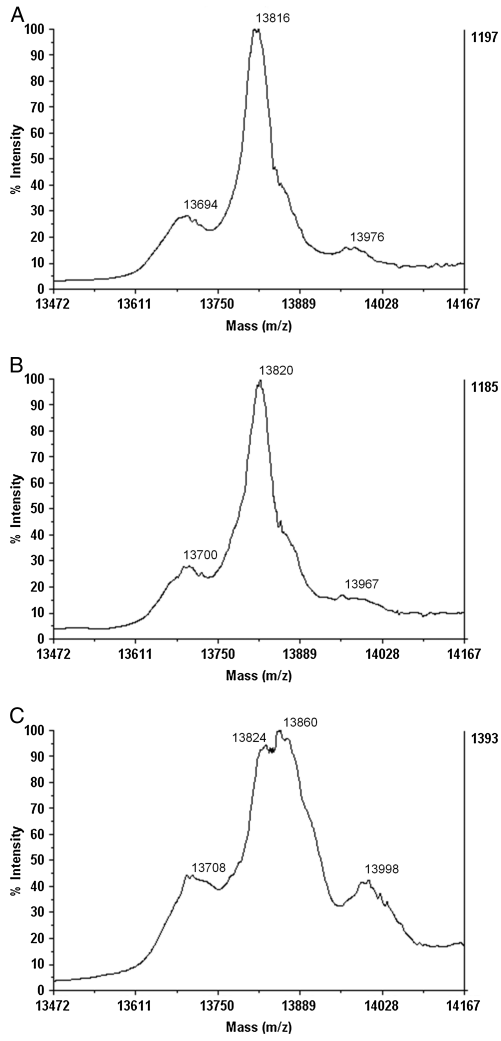


**Figure 1.** MALDI-TOF-MS spectra of the serum samples of a liver transplant donor (A), negative (B), and positive (C) controls.

possible benefits and harms. Nonetheless, as the whole point of transplanting an FAP patient is to give her/him a wild type TTR producing liver, screening of the donor should perhaps be given serious consideration.

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# Inability of Mutant Transthyretin V30M to Cross the Blood-Eye Barrier

Liver transplantation is the main therapeutic option for familial amyloidotic polyneuropathy (FAP) (Portuguese type, caused by a substitution of methionine for valine at position 30 of transthyretin - TTRV30M) (*1*). It virtually eliminates circulating TTR V30M, with a positive impact on survival and quality of life (*2*). Domino liver transplantation using grafts from FAP patients is a well-established procedure (*3*) and is regarded as a safe way to increase donor availability (*4, 5*). However, reports of de novo amyloidosis in FAP liver recipients (*6, 7*) have caused much concern.

FAP patients present ophthalmic disorders such as lacrimal dysfunction, pupillary disturbances, glaucoma, and vitreous opacities (*8–10*). The ability of mutant transthyretin to cross the blood-brain (*11*) and blood-nerve barriers (*6*) has been documented, but no conclusive data exists about its ability to cross the blood-eye barrier. If the circulating TTR V30M penetrates the eye, the possibility of acquired amyloidotic oculopathy should be considered in FAP liver recipients.

## CASE REPORT

A 63-year-old male, submitted to domino liver transplantation because of hepatic alcoholic cirrhosis 2 years previously, underwent cataract surgery. No previous ocular disease was reported. At the beginning of surgery, 0.1 mL of aqueous humor was collected from the anterior chamber and used for TTR V30M detection by cyanogen bromide cleavage and sodium dodecyl sulfate–polyacrylamide gel electrophoresis, as described by Saraiva et al. (*12*), followed by Western blot analysis with antiprealbumin primary antibody (FL-147, #sc-13098; Santa Cruz Biotechnology, CA). As positive and negative controls, we used sera from two individuals with a definite diagnosis based on clinical findings and the result of molecular testing for the TTR V30M mutation. Cleavage of TTR V30M with cyanogen bromide produces an additional 10.6-kDa peptide fragment because of the methionine residue at position 30. This fragment was detected in the serum of both the positive control and the domino liver transplant recipient (Fig. 1A,B). In the aqueous humor of the domino liver transplant recipient, this band was absent.

These results were complemented by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometer (MALDI TOF-MS) analysis, as described by Haraoka et al. (*13*). Mass spectra of TTR immunoprecipitated from the aqueous humor and serum samples were acquired in a 4800 MALDI-TOF/TOF-MS (Applied Biosystems, Foster City, CA) and were analyzed with Data Explorer software version 4.9 (Applied Biosystems). The spectra obtained are represented in Figure 1C–F. Taking as reference previously published results (*13*), we can identify three peaks for TTR in the positive control sample that correspond to free and cysteine (Cys)-conjugated forms of wild-type TTR (13,776±10 d and 13,890±10 d) and Cys-conjugated form of the TTR V30M variant (13,923±10 d). In the negative control sample we can see only the peaks of the free and Cys conjugated forms of wild-type TTR (13,766±10 d and 13,880±10 d). As in the negative control sample, the spectrum of the aqueous humor of the FAP liver transplant recipient has the two peaks attributed to wild-type TTR (13,761±10 d and 13,883±10 d), but the peak of mutant TTR is absent. As expected, the mutant TTR V30M peak was detected in this patient's serum (13,911±10 d).

## DISCUSSION

Amyloid deposition has an important role in oculopathy in FAP patients. Therefore, determining the ability of mutant TTR to cross the blood-eye barrier is essential to predict the possible future occurrence of endocular pathology in FAP donor domino liver recipients.

In this study, mutant TTR was not detected in the aqueous humor of an FAP liver recipient despite its presence in the serum, suggesting that TTR and, in particular, TTR V30M is unable to cross the blood-eye barrier. Thus, the risk of severe endocular disease seems to be negligible in FAP liver recipients. Ocular manifestations associated with extraocular amyloid deposition may still occur.

This study was carried out in a liver transplant recipient with no known previous eye disease. We do not know if, in patients with existing or supervening conditions, the integrity of the blood-eye barrier is maintained.

It might be argued that small quantities of variant TTR could be present in the aqueous humor at a concentration below the detection limits of both the Western blotting and MALDI-TOF assays used. However, even if that was the case, we would like to point out that protein aggregation and fibrillogenesis are highly concentration-dependant phenomena and that a threshold exists below which aggregation is unlikely to occur (*14, 15*). This work reinforces the current consensus that domino liver transplantation justifies the risks of iatrogenic FAP, although careful monitoring of these patients will be necessary.

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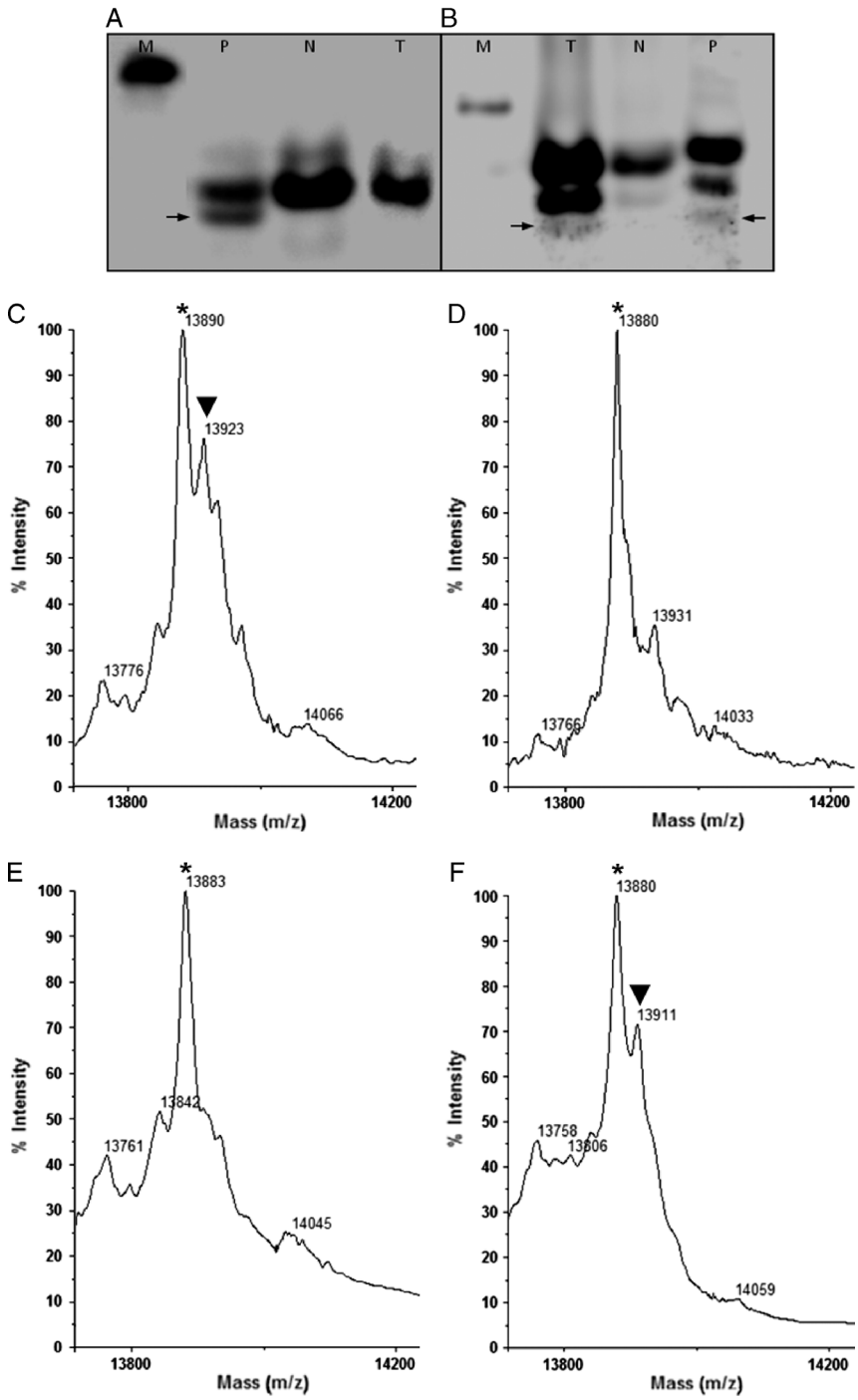


**FIGURE 1.** Western blot after cyanogen bromide cleavage (A and B) and mass spectra (C–F); fragment (*arrow*) containing the residues 30–127 of the transthyretin with replacement at position 30 methionine for valine (TTR V30M) polypeptide chain, present only in the carriers of the mutation. Lane P represents the positive control serum; N, the negative control serum; T, the aqueous humor from the transplanted patient (A) and serum from the transplanted patient (B). Lanes M represent the molecular weight marker used (MagicMark; Invitrogen, Carlsbad, CA). Mass spectra of a positive (C) and negative (D) control serum for the TTR V30M mutation and the aqueous humor (E) and serum sample (F) of the transplanted patient. * Cysteine-conjugated form of wild-type TTR; ▼ Cysteine-conjugated form of amyloydosis TTR V30M.

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# New-Onset Parkinson Syndrome After Liver Transplantation

Serious neurologic complications (NCs) may occur in up to 45% of patients after liver transplantation (LT). The most common NCs were considered to be encephalopathy and seizures in many published series (*1*) and were associated with increased morbidity and mortality (*2*). It is often difficult to identify the cause of the neurologic symptoms because of the unspecific nature of their presentation. NCs are frequently attributed to the well-described adverse effects of calcineurin inhibitors, tacrolimus (TAC), or cyclosporine A (*3*). The failure to recognize and adequately treat NCs can lead to a fatal outcome (*4*).

In this case report, we describe a manifestation of Parkinson syndrome after LT.

A 47-year-old female underwent deceased donor LT caused by alcohol-related liver cirrhosis. The surgery was uneventful. The immunosuppression therapy was a calcineurin inhibitor–based triple regimen with TAC (with a target level of 5–8 ng/mL), mycophenolate mofetil, and prednisone. The liver function tests were in the reference range within 1 week. The patient was able to be weaned from the ventilator 5 hr after surgery. At this time, she was fully awake and had good motor function. There were no dopamine-blocking agents used.

On postoperative day (POD) 1, the patient seemed somnolent. She followed commands but was unable to talk or move voluntarily. Slight rigor in the patient's extremities was also noted. On the same day, the patient developed akinesia, which significantly compromised her respiratory function. In the evening of POD 1, the patient was reintubated. Her TAC blood level at this time was 8.2 ng/mL. A computed tomographic scan of the head did not reveal any pathologic findings. On POD 2, the patient recovered completely and was extubated. A neurologic consult was performed, and no deficits were found; however, 12 hr later, the patient's condition deteriorated significantly. The patient developed the same clinical picture and had to be reintubated but could be extubated few hours later. On POD 3, the same clinical scenario repeated again. The neurologist was able to see the patient before reintubation and gave a diagnosis of an atypical Parkinson syndrome. Levodopa and benserazide were administered. Soon after treatment was started, the symptoms resolved, and she finally maintained respiratory stability. On POD 15, the patient was discharged from the intensive care unit. A follow-up magnetic resonance imaging performed on POD